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Novel Sample Preparation and TOF-MS Analysis of Environmental and Toxicological Analytes Using EPA Method 6800

Rebecca Wagner

NOVEL SAMPLE PREPARATION AND TOF-MS ANALYSIS OF ENVIRONMENTAL AND TOXICOLOGICAL ANALYTES USING EPA METHOD 6800

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By

Rebecca L. Wagner

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H.M. Skip Kingston, Ph.D. Professor of Chemistry and Biochemistry (Committee Chair)	Ellen S. Gawalt, Ph.D. Associate Professor of Chemistry and Biochemistry (Committee Member)
Stephanie J. Wetzel, Ph.D. Assistant Professor of Chemistry and Biochemistry (Committee Member)	Frederick Fochtman, Ph.D. Director of Forensic Science and Law (Committee Member)
David W. Seybert, Ph.D. Dean, Bayer School of Natural and Environmental Sciences Professor of Chemistry and Biochemistry	Ralph A. Wheeler, Ph.D. Chair, Department of Chemistry and Biochemistry Professor of Chemistry and Biochemistry

ABSTRACT

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The quantitative analysis of environmental and toxicological samples must be reliable, rapid, and in some cases field portable. In the United States, the employment of chemical weapons by rogue states and/or terrorist organizations is an ongoing concern. Nerve agent degradative products (methylphosphonic acid) as well as surrogates (glyphosate) must be detected at low quantities in various water matrices. Current methods describe tedious and time-consuming derivatizations for gas chromatography-mass spectrometry and liquid chromatography in tandem with mass spectrometry. Two solid phase extraction (SPE) techniques for the analysis of glyphosate and methylphosphonic acid are described with the utilization of isotopically enriched analytes for quantitation using

atmospheric pressure chemical ionization-quadrupole-time of flight-mass spectrometry (APCI-Q-TOF-MS) that does not require derivatization.

The use of illicit drugs is also an increasing problem in the United States. Toxicological analysis of illicit drugs is important for death investigation as well as in the treatment of individuals whom abuse and misuse drugs. This dissertation describes a newly developed analytical method for the quantitative analysis of heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl simultaneously in synthetic urine. The resolution of an electrospray ionization-time of flight-mass spectrometer (ESI-TOF-MS) was utilized for simultaneous analysis of the drugs after extraction from urine using two newly developed SPE procedures.

The first SPE technique described in this dissertation is solid phase extraction-isotope dilution mass spectrometry (SPE-IDMS). It involves applying EPA Method 6800 by pre-equilibrating a naturally occurring sample with an isotopically enriched standard prior to SPE. The second extraction method, i-Spike, involves loading an isotopically enriched standard onto a SPE column independently from the naturally occurring sample. The sample and the spike are then co-eluted from the column enabling precise and accurate quantitation by molecular IDMS calculations. The SPE methods in conjunction with IDMS eliminate concerns of incomplete elution, matrix and sorbent effects, and MS drift. For accurate quantitation with IDMS, the isotopic contribution of all atoms in the target molecule must be statistically taken into account. This dissertation describes two newly developed sample preparation techniques for the analysis of environmental and

toxicological samples as well as statistical probability analysis for accurate molecular IDMS.

DEDICATION

To my fiancé, Cory M^{c}Kissick, and to my parents, Ron and Sue Wagner, I wouldn't have made it if it weren't for you.

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ppm	parts per million
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
LC-MS/MS	liquid chromatography tandem mass spectrometry
ICP-MS	inductively coupled plasma-mass spectrometry
ppb	parts per billion
SPE	solid phase extraction
TOF-MS	time of flight-mass spectrometry
CWC	chemical weapons convention
LD_{50}	lethal dose of fifty percent of the population
LLE	liquid-liquid extraction
ESI-TOF-MS	electrospray ionization-time of flight- mass spectrometry
IDMS	isotope dilution mass spectrometry
IDMS/SIDMS	isotope dilution mass spectrometry/speciated isotope dilution mass spectrometry
ESI	electrospray ionization
APCI	atmospheric pressure chemical ionization
MALDI	matrix assisted laser desorption/ionization
APPI	atmosphere pressure photoionization
TOF	time-of-flight
PAH	polycyclic aromatic hydrocarbon

USEPA	United States Environmental Protection Agency
LC-MS	liquid chromatography-mass spectrometry
EPF	error propagation factor
APCI-QQQ-MS	atmospheric pressure chemical ionization-triple quadrupole-mass spectrometry
APCI-Q-TOF-MS	atmospheric pressure chemical ionization-quadrupole-time of flight-mass spectrometry

Chapter 1

Introduction

1.1 Current trends in the analysis of glyphosate

Glyphosate, a nonselective post-emergent herbicide, is extensively used in the United States for total vegetation control.[1-4] In the mid 1990's, the advent of genetically modified glyphosate-resistant crops increased the utilization of glyphosate allowing it to become the most widely utilized herbicide in the world.[5] Although its popularity only became global in the 1990's, glyphosate has been extensively used in the United States since its introduction in 1974.[5-9] It is the active ingredient in many commercially available herbicides such as Roundup, Rodeo, and Touchdown. In the United States, 31% of all planted corn acres were treated with glyphosate in 2005 as well as 92% of all planted soybean acres in 2006.[10] Due to its extensive applications of use, glyphosate is now on the list of the United States national primary drinking water contaminants with a maximum contaminant level goal of 0.7 parts per million (ppm).[5, 9, 11]

Genetically modified glyphosate-resistant crops have increased the use of glyphosate and subsequently the pollution of the herbicide in rivers and surface waters.[12] The contamination of waterways causes contamination in humans from food, feed, and contaminated ecosystems.[13-14] Although plants are genetically designed to handle high levels of these herbicides, the food chain is still impacted in the ingestion of the genetically modified contaminated plants. It is often suggested that the toxicity of glyphosate is extremely low but the toxic threshold *in vivo* is actually still unknown.[15]

Gasnier *et al.* demonstrated that part per million levels of glyphosate caused DNA damage and cytotoxic effects on endocrine disruptors in human cell lines.[15]

Glyphosate is a highly polar organophosphate molecule containing four pK_a values of 0.7, 2.6, 5.6, and 10.6. It contains a highly ionized phosphate group, a secondary amine group, and a carboxylic acid group.[16] The chemical structure as well as the pK_a values are demonstrated in Figure 1.1.

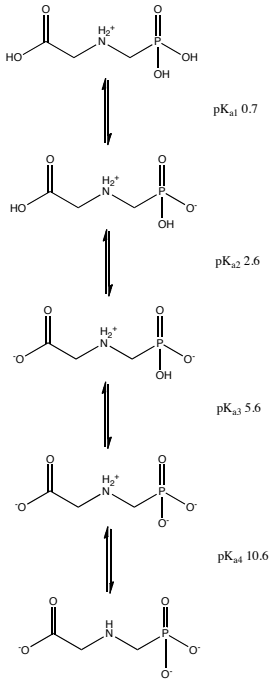


Figure 1.1 Glyphosate structure with pKa values

The high polarity and high ionic character causes insolubility in organic solvents and low volatility making this ampohteric molecule difficult to detect in analytical instruments.[9,16] The analysis of glyphosate via gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) requires tedious and time-consuming derivatizations due to the polar and ionic character of the analyte.[1-2, 8, 17-20] For GC-MS analysis derivatization is required to decrease the polarity of the molecule and increase

volatility. On the other hand, HPLC analysis requires derivatization due to the lack of a chromophore or fluorophore for detection.[9]

More recent methods have been developed using liquid chromatography tandem mass spectrometry (LC-MS/MS) techniques. Unfortunately these methods often times still require derivatization for column retention.[2-3, 21-24] Inductively coupled plasma-mass spectrometry (ICP-MS) coupled with ion chromatography is often utilized in the analysis of water quality samples and other environmental samples.[5, 9] This analysis is advantageous due to the part per billion (ppb) limit of quantitation levels achieved.[9] A drawback for the utilization of ICP-MS for the accurate quantitation of glyphosate in drinking water samples is that ICP-MS offers only elemental analysis. Therefore, the quantity of glyphosate would simply be determined by the quantitation of phosphorous in the sample.

Chapter 3 of this dissertation describes newly developed methods for the analysis and quantitation of glyphosate in drinking water samples. The methods involve a solid phase extraction (SPE) with the employment of isotope dilution mass spectrometry (IDMS) for quantitation using time of flight-mass spectrometry (TOF-MS). These methods meet the goals of glyphosate analysis, which are a rapid and reliable method that does not require derivatization but still maintains low limits of quantitation.

1.2 Current trends in the analysis of methylphosphonic acid

The identification of chemical weapons is essential for the compliance with the Chemical Weapons Convention (CWC). Although the CWC prohibits the development, production,

stockpiling, and use of chemical weapons the threat of use with rogue states and terrorist organizations is still viable.[25] The lethal dose for fifty percent of the population (LD_{50}) for these agents ranges from 0.69 ppm to 2.0 ppm after only two minutes of exposure.[26] Organophosphate nerve agents inhibit hydrolysis of the neurotransmitter acetylcholine within nerve synapses by reacting with the serine residue in the active site of acetylcholinesterase. The loss of acetylcholinesterase activity initiates an accumulation of acetylcholine which results in over stimulation and eventual paralysis of muscles.[27-28] Nerve agents are a class of a chemical warfare agent that disrupts neurological regulation by means of inhibiting acetylcholinesterase.[29-31] The release of nerve agents cause mass chaos and death but it will also instill fear into society, which meets the goals of many terrorist organizations. For example, in Japan, in 1994 and 1995, organophosphate nerve agents were disseminated in the mass transit system.[27]

Phosphorus containing compounds are present as commercially available pesticides and pose a threat to public as potentially fatal nerve agents. Glyphosate is being utilized as a surrogate for G and V series nerve agents due to the similarities in chemical structure. G series nerve agents were developed in 1930 when Nazi Germany stockpiled tabun and sarin during World War II.[26-27] V series nerve agents were developed in the early 1950's by Imperial Chemicals Industries Limited in Britain. Environmental exposure causes nerve agents to rapidly hydrolyze into alkyl methylphosphonic acids and methylphosphonic acid in low ppb quantities.[32-33] Phase one hydrolysis products of VX and GB are ethyl methylphosphonic acid and isopropyl methylphosphonic acid

respectively, which are both water soluble, polar, and acidic in nature.[32, 34-35] Figure 1.2 represents the degradation of VX and GB into methylphosphonic acid.

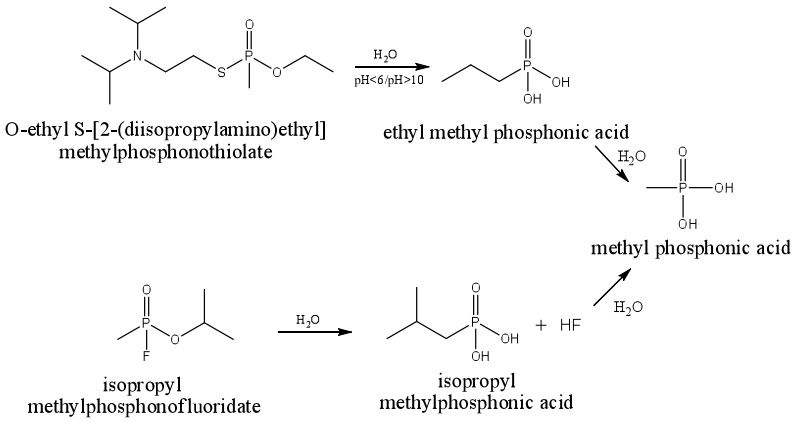


Figure 1.2. Degradative process of VX and GB nerve agents in the presence of water

Due to the stability of the alkyl methylphosphonic acids when compared to their corresponding nerve agent, they are commonly utilized for indicating the presence or utilization of organophosphate nerve agents.

Detection of these hydrolysis products is difficult because they lack a chromophore or fluorophore for UV or fluorescence detection.[36] The detection and quantitation of nerve agents must be coupled with the analysis of their degradation products for verification of use.[29, 37] The hydrolysis of V series and G series nerve agents produce methylphosphonic acid that has a decreased toxicity compared to the original agents.[38] Methylphosphonic is an amphoteric molecule with two pK_a values as shown in Figure 1.3.

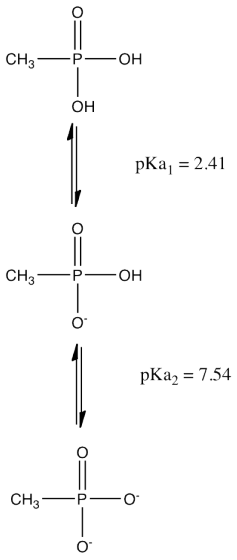


Figure 1.3. Methylphosphonic acid chemical structure with pKa values

Current techniques for the detection of nerve agents includes analysis by GC-MS[31,39], LC-MS[30, 37, 40-41], and ion chromatography.[38] There are many drawbacks when using GC-MS, which include sample clean up and a tedious derivatization process prior to analysis. Derivatization typically requires the addition of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetaminde (MTBSTFA) to the sample and an incubation period of one hour at 100 °C. The sample is then evaporated to dryness under a slow stream of nitrogen before reconstituting the derivatized analyte is ethyl acetate for analysis. LC-MS analysis is expensive and difficult to operate for a novice individual while ion chromatography has a low specificity and sensitivity. The use of a calibration curve or internal standard for quantitation is also a drawback with all three techniques described above. There are many sources of error when using these quantitative techniques. Some examples include having a different matrix for the analyte and standard, matrix effects, loss of sample, physical and chemical interferences/differences, and instrument drift.[42]

Chapter 4 describes the methods developed for the analysis of methylphosphonic acid in water samples. These newly developed methods were an extension of the SPE analysis of glyphosate in drinking water samples using TOF-MS. The methods described in this chapter are viable for the analysis of glyphosate and methylphosphonic acid simultaneously enabling a multidimensional analysis for agricultural and weapons of mass destruction applications.

1.3 Current trends in the toxicological analysis of illicit drugs

In 2009, an estimated 21.8 million Americans aged 12 or older were current illicit drug users, which is described as the use of marijuana/hashish, cocaine (including crack), heroin, hallucinogens, inhalants, or prescription-type psychotherapeutics used non-medically within the past month.[43] Of the 21.8 million Americans, 9.2 million were users of illicit drugs other than marijuana/hashish.[43] The increasing use of illicit drugs affects not only the drug related emergency department visits but also increases the drug related mortality rate. In 2009, there were approximately 4.6 million emergency department visits that were associated with misuse and abuse of drugs including 1.0 million of those cases involving illicit drugs.[44] This estimate is higher than the 1.9 million emergency department visits associated with drug misuse or abuse in 2007.[45] The highest of the illicit drug rates include cocaine, heroin, and marijuana with rates of 422,896; 213,118; and 376,467 people respectively.[44] Most emergency department visits and mortality rates due to drug misuse are from the use of multiple drugs. In 2006, the analysis of area profiles where drug misuse caused death revealed that over 50 percent of the cases involved more than one drug within the states of Maine, New Hampshire, Vermont, Maryland, Utah, and New Mexico.[46]

Opiates and/or opioids are described as any natural or synthetic drug with morphine like properties.[47] The classification of opiates includes opium, morphine, diacetylmorphine, methadone, and codeine. Heroin, a semi synthetic morphine derivative, was developed in 1874 by A.C. Wright to increase the potency of morphine.[48-50] The increased potency is due to its higher lipopholicity compared to morphine enabling heroin to cross the blood brain barrier at a faster rate.[50-51] Heroin is rapidly metabolized by serum and liver esterase activity into 6-actylmorphine and subsequently morphine. The clearance of morphine occurs via glucuronidation in the liver, predominantly to morphine-3-glucuronide and morphine-6-glucuronide.[52-55] The elimination half-life of heroin is between two and eight minutes.[48-50, 55] Due to the rapid metabolism of heroin, unequivocal proof of heroin use requires the identification of 6-acetylmorphine in blood or urine.[56] The half-life of 6-acetylmorphine is between six and twenty-five minutes in blood and eight hours in urine.[49-50, 52, 57] In postmortem cases, metabolism continues in blood and is stable in urine. 6-Acetylmorphine is then metabolized to morphine, which possesses an elimination half-life of 40 minutes.[55, 57] The metabolism of heroin is depicted in Figure 1.4.

Papaver *samniferum* poppies

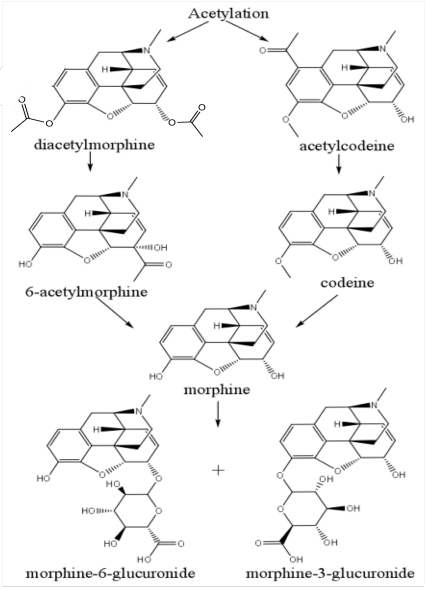
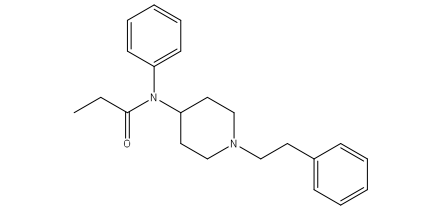


Figure 1.4. Metabolism of heroin and acetylcodeine from the Papaver samniferum poppy

Detection of opiates not only includes the detection of the drug itself but also its metabolites for unequivocal identification of the abused drug. Another aspect of illicit drug use that complicates drug identification is the use of contaminants or combination drug use. Contaminants are components that are added to the illicit drug to either increase or decrease the effect of the main drug component.[58] Contaminants are also used as "bulking-agents" to increase the quantity sold but decreasing the overall amount of illicit drug present. In 2006, the Philadelphia Medical Examiner's Office reported the detection of xylazine and fentanyl in drug-related cases. Also in 2006, there were several cities in the United States that reported increased mortality rates due to fentanyl. These cases were determined to be cocaine and heroin samples that were "contaminated" with fentanyl.[59] Fentanyl is used medically in the treatment of severe chronic pain and is estimated to be 30-50 times more potent than heroin.[59] The structure of fentanyl is shown

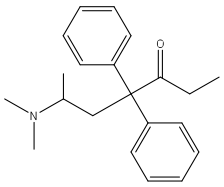
in Figure 1.5 Other than fentanyl common contaminants include heroin, cocaine, codeine, procaine, and quinine.[58]



N-(1-(2-phenylethyl)4-piperidinyl)-N-phenyl-propanamide
Fentanyl

Figure 1.5. Chemical structure of fentanyl

A program subject involvement in drug treatment therapy may also complicate the analytical detection of illicit drugs in urine. Methadone, shown in Figure 1.6, has been one of the most widely used drugs to treat withdrawal symptoms associated with opiates since 1950.[60] Often times even if a patient is associated with a methadone treatment program, they continue to use illicit drugs. Fatalities due to methadone use greatly increased in the early 2000's. The majority of methadone related deaths included the use of another opiate or central nervous system depressant.[60]



6-(dimethylamino)-4,4-diphenylheptan-3-one
Methadone

Figure 1.6. Chemical structure of methadone

Although methadone treatment programs are the most common form of opiate dependence treatment in the United States, a few European countries have pharmaceutical heroin treatment programs for heroin addiction. This treatment program is for individuals that fail to respond to the more traditional methadone treatment.[6] This

imposes yet another layer into the complicated process of illicit drug quantitation. Pharmaceutical heroin possesses the same metabolites in blood and urine as illicit heroin. Therefore, the identification of impurities in the illicit production of heroin must be identified to provide information on illicit heroin use while in a pharmaceutical heroin treatment program. One of the most common impurities in heroin production is acetylcodeine, which has been reported to be present in 86% of urine samples containing illicit heroin.[6, 61]

The qualitative and quantitative analysis of illicit drugs in urine not only affects the treatment of patients in emergency departments but impacts death investigation in both the private and public sectors. The analyses must be comprehensive to cover a wide range of illicit drugs, metabolites, common contaminants, and even drugs used in treatment for illicit drug use. Currently analytical techniques for the analysis of illicit drugs include GC-MS and LC-MS/MS.

Current analysis protocols describe initial screening of illicit drugs by immunological methods.[62-63] Immunological methods are limited in their screening for a wide range of drugs, high cost of reagents, and high incidence of false negatives.[62, 64-65] These limitations are a significant downfall in the current detection of illicit drugs. Currently, if a positive immunological test is not present the sample does not progress into analytical analysis and identification. Although these techniques are sensitive, if the test does not screen for the drug that is present, a negative result ensues and the sample does not go onto analytical identification.

Positive immunological samples undergo quantitative analytical analysis. Blood and urine samples are typically quantitatively assessed by internal standard or calibration curve techniques.[58-59, 66-67] Most analysis techniques require a sample clean up step to remove matrix interferences. These techniques include SPE, liquid-liquid extraction (LLE), and protein precipitation.[58-59, 65-66, 68-70] GC-MS analysis of illicit drugs was the gold standard until the advent of LC-MS/MS.[71-73] The analysis of illicit drugs by GC-MS typically involves a tedious and time consuming process of sample clean up followed by analyte derivatization to increase volatility for analysis.[51, 62, 64, 66-68, 74-75] The derivatization procedure traditionally includes the addition of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) to the sample and enabling the derivatization to occur for 30 minutes to one hour at 70 °C. After derivatization the samples are evaporated to dryness under nitrogen and reconstituted with ethyl acetate for analysis. Unlike GC-MS methods, LC-MS/MS methods do not require derivatization of the illicit drugs prior to analysis and are typically analyzed in selected ion monitoring mode to increase sensitivity.[47, 64, 71] LC-MS/MS still requires sample preparation and matrix effects of samples must still be accounted for.[64, 68, 71] Other advantages of LC-MS/MS over the traditional GC-MS analysis are that the sample can be analyzed in a scan mode or a multiple reaction monitoring mode to select for ions and identify parent ions.[69]

Although LC-MS/MS is a selective technique, quantitative identification may become difficult with matrix interferences and a large quantity of analytes to identify. A

simultaneous method for the quantitative analysis of heroin, 6-acetylmorphine, morphine, cocaine, codeine, fentanyl, and methadone in urine was developed using SPE followed by analysis on an electrospray ionization-time of flight-mass spectrometer (ESI-TOF-MS). Quantitative assessment was completed using IDMS.[42] IDMS in conjunction with ESI-TOF-MS will enable greater resolution and increased accuracy compared to the current GC-MS and LC-MS/MS techniques. The resolution on the TOF-MS is significantly greater than the unit mass resolution of the LC-MS/MS decreasing the opportunity of interferences from matrix.[76]

1.4 Solid phase extraction

The development of analytical methods for the analysis of trace level analytes involves numerous steps from sample collection to final data analysis. One of the most important steps within method development is the sample preparation. Sample preparation can include LLE and SPE to 'clean up' and concentrate the target analytes. As well as being the most important step, it is often times the most time consuming and tedious. It is also where the most imprecision and inaccuracy is introduced within a measurement.[77]

The solid phase extraction sample preparation technique was established in 1978 and is now one of the most widely used techniques for the isolation of selected analytes.[78-79] SPE is advantageous over the use of a LLE because it not only enables clean up but also preconcentration. Other advantages of SPE are that is requires little solvent, small sample volumes, is less time consuming, and has the potential to be automated.[77]

SPE employs the utilization of a small cartridge that contains specific packing materials that is packaged between two fritted disks. The packing can be a wide range of material depending on the chemical characteristics of the analyte and its matrix. The packing consists of a backbone that can be either silica or polymeric and a chemically bonded functional group. There are advantages and disadvantages when using silica or polymeric backbones. Polymer phases can be used over the entire pH range but the conditioning of the cartridge is more time consuming than a bonded silica backbone.[77]

Method development of a SPE extraction procedure includes choosing the packing material and backbone as well as developing the protocol. The extraction process occurs typically in five steps. The first step involves activating the SPE sorbent packing by passing through solvent. The second step involves removing the solvent and conditioning the SPE sorbent with a solvent that has similar chemical characteristics to the analytes matrix. The third step is to apply the sample to the column. The analyte is retained by the sorbent and the remaining matrix will flow through the sorbent and be discarded. The fourth step involves removing any interfering compounds and the final step is the elution of analytes.

The SPE for glyphosate and methylphosphonic acid in water were developed in Chapter 3 and Chapter 4 using a Phenomenex Strata-SAX strong anion exchange column. The SPE sorbent consists of a silica backbone with a propyl quaternary amine functional group with a chloride counter ion. During extraction, the negatively charged glyphosate and methylphosphonic acid displace the chloride counter ion and are retained by the

column via electrostatic interactions. The porosity and pH stability are vital to the SPE of the glyphosate and methylphosphonic acid. The porosity of the silica provides the necessary surface area for rapid extraction and allows small molecules to enter the pores and be retained by the electrostatic interactions between the quaternary amine and negatively charged analyte.

The SPE for illicit drugs in synthetic urine was developed using a United Chemical Technologies Clean Screen CSDAU SPE column. The bonded silica backbone contains two functional groups for the extraction of non-polar and ionic analytes. The two functional groups include a reverse phase and an ion exchanger. In this case the ion exchanger is a benzenesulfonic acid. During extraction, the acidic, basic, and neutral drugs interact with the SPE column due to polarity and charge of the analytes. The utilization of a mix mode SPE sorbent enables a wider range of analytes to be extracted from the synthetic urine matrix.

The advantages of SPE in conjunction with accuracy and precision of IDMS and the resolution of TOF-MS were utilized to develop optimal procedures of the extraction/preconcentration of phosphonic acid containing compounds in water and seven illicit drugs in urine samples. The disadvantages of SPE include the surface chemistry, mixed retention mechanisms, and analyte recoveries are all accounted for with the utilization of an isotopically enriched analogue of the analyte and IDMS quantitation.

1.5 Isotope dilution mass spectrometry

EPA Method 6800, isotope dilution mass spectrometry and speciated isotope dilution mass spectrometry (IDMS/SIDMS) is a quantitative analytical technique based on the measurement of isotopic ratios in a sample that has been spiked with a known amount of an isotopically enriched compound. The relationship between the naturally occurring analyte and the isotopically enriched spike are mathematically expressed using the isotope dilution equation.[42] Traditionally, IDMS quantitation is utilized in the field of elemental speciation and the analysis of metals.[80-83] It is considered a definitive method for trace element analysis.[84] The International Bureau of Weights and Measures regards IDMS as a primary ratio method of the highest metrological quality.[84] IDMS has the ability to be applied to the analysis of organic compounds given the availability of different isotopically labeled analogues of the compounds.

Isotope dilution is often times regarded as the only quantitative method system in which analyte formation occurs during the analysis process.[81] Another commonly stated advantage is that upon equilibration, any loss of analyte is insignificant.[85] It can correct for any matrix effects and partial analyte loss that may occur during sample preparation and has the ability to quantitatively assess the transformation between two species that cannot otherwise be determined by traditional quantitative methods.[84]

To ensure unbiased results using IDMS, there are a number of prerequisites that must be addressed when choosing an isotopically enriched analyte. The analyte must be well defined. The isotope must be stable and enriched at a known purity that also has identical

behavior to the analyte. Finally, sampling must be representative between the analyte and spike. Meaning that full equilibration must occur between both the analyte and spike prior to analytical analysis. IDMS techniques and calculations are well established for elemental analysis, but molecular analysis is still in its infancy.[84]

Upon mass spectral analysis, ratio analysis is completed with the naturally occurring sample and its isotopically enriched spike. This ratio is then utilized for the IDMS equation depicted in Equation 1.1.

Equation 1.1
$$Concentration, \mu g/g = \left(\frac{W_{sp} \bullet C\left[A_{sp} - R \bullet B_{sp} \right]}{\left(B \bullet R \right) - A} \right) \bullet \left(\frac{M}{W_s} \right)$$

The IDMS equation takes into account the weight of the isotope spike in the solution (W_{sp}), concentration of the spike (C), the atomic fraction of the isotope A in spike (A_{sp}), the atomic fraction of isotope B in spike (B_{sp}), the atomic fraction of isotope A in sample (A), and the atomic fraction of isotope B in sample (B). Other coefficients that are taken into account in the IDMS/SIDMS mathematical equation are the atomic weight of the analyte (M), the weight of the sample (W_s), and the experimentally measured isotope ratio of A/B (R).

A variation of IDMS is speciated isotope dilution (SIDMS), which takes into account any transformation of species. In SIDMS each species is isotopically labeled enabling the interconversions between the species occurring after spiking the isotopically enriched analytes to be accounted for and mathematically corrected.[42, 86] In the case of the analysis of illicit drugs, not only can the metabolism of heroin in urine be tracked and corrected for, but the original concentration of heroin can be determine. This is an important aspect

in death investigation for drawing conclusions on the cause of death. In some cases, the combination of drugs or even contaminants may have caused the death, not the illicit drug. This is also an important quantitative technique for the public health sector for the treatment of illicit drugs to determine which illicit drug was taken and the concentration of the drug itself. No other method currently available is capable of providing legally defensible, accurate, and precise quantitative analysis of species and metabolite transformation simultaneously.

The SIDMS mathematical calculation can be used to follow and quantify the interconversions/metabolism process that may occur between heroin, 6-acetylmorphine, and morphine. The interconversion is a unidirectional metabolism where heroin metabolizes to 6-acetylmorphine and subsequently 6-acetlymorphine metabolizes to morphine. For example the unidirectional transformation of a sample containing species of *Z*, and the species are *K* and *L*, with concentrations of C_X^K (μmol/g) and C_X^L (μmol/g), respectively. Weigh W_X gram of the sample, followed by the addition of W_S^L grams of ${}^F K$ spike (species *K* enriched with isotope "F") and W_S^L grams of ${}^G L$ spike (species *L* enriched with isotope "G") into the sample. After spiking, the sample contains ${}^F A_X C_X^K W_X + {}^F A_S^K C_S^K W_S^K$ μmoles of ${}^F Z$ as *K* and ${}^F A_X C_X^L W_X + {}^F A_S^L C_S^L W_S^L$ μmoles of ^{F}Z as *L*, where *A* represents the isotopic abundance. The unidirectional transformation has a fraction of *K* that converts to *L* which is designated as α. Since no *L* converts to *K*, β will be zero. The value for N_X^K can be calculated from Equation 1.2, which is the simple IDMS equation. Where N_X^K is the number of moles of species, *K*.

Equation 1.2

$$N_X^K = \frac{N_S^K\left({}^F A_S^K - R_{F/R}^K \bullet {}^R A_S^K\right)}{\left(R_{F/R}^K \bullet {}^R A_X - {}^F A_X\right)}$$

The value of N_X^K can then be substituted into Equation 1.3 to solve for N_X^L and α, where N_X^L is the number of moles of species *L* and α is the fraction of *K* that converts to *L*.

Equation 1.3

$$N_X^L = \frac{\left(C_3 B_4 - C_4 B_3\right)}{\left(A_3 B_4 - A_4 B_3\right)} \quad \text{and} \quad \alpha = \frac{\left(A_3 C_4 - A_4 C_3\right)}{\left(A_3 B_4 - A_4 B_3\right)}$$

Where,

$$A_3 = \left(R_{F/R}^L {}^R A_X - {}^F A_X\right)$$
$$B_3 = \left[R_{F/R}^L\left({}^R A_X N_X^K + {}^R A_S^K N_S^K\right) - \left({}^F A_X N_X^K + {}^F A_S{}^K N_S^K\right)\right]$$
$$C_3 = N_S^L\left({}^F A_S^L - R_{F/R}^L {}^R A_S^L\right)$$

and

$$A_4 = \left(R_{G/R}^L {}^R A_X - {}^G A_X\right)$$
$$B_4 = \left[R_{G/R}^L\left({}^R A_X N_X^K + {}^R A_S^K N_S^K\right) - \left({}^G A_X N_X^K + {}^G A_S^K N_S^K\right)\right]$$
$$C_4 = N_S^L\left({}^G A_S^L - R_{G/R}^L {}^R A_S^L\right)$$

The utilization of IDMS and SIDMS for quantitation of both the environmental and toxicological samples enables a more precise and accurate quantitation method when compared to calibration curve or internal standard methods of quantitation. Traditionally, IDMS/SIDMS are employed for elemental analysis. Chapter 2 discusses the extension of traditional IDMS to molecular compounds. The analysis of a molecular species requires significantly more statistical analysis to determine the constant variables in the IDMS equation. This is due to the fact that not only is there a contribution of the isotopically enriched species to the natural species but also a natural probability of the other atoms in the molecule to contribute both to the naturally occurring peak and isotopically enriched peak in the mass spectra. With the corrections applied for IDMS quantitation of molecules, a more reliable quantitation method can be employed for analysis of countless molecules.

1.6 Time-of-flight mass spectrometry

Time-of-flight mass spectrometry has become a widely used mass analyzer due to the high resolving power associated with mechanisms of ion separation in the flight tube. In order for analysis in the TOF-MS, the sample must first be ionized for detection. In recent years numerous ionization methods have been developed including electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption ionization (MALDI), and atmospheric pressure photoionization (APPI). The two ionization sources that are focused on in this dissertation are ESI and APCI.

John Fenn whom later won the 2002 Nobel Prize for his invention, developed ESI in the late 1980's.[87] ESI is considered to be a soft ionization technique that enables the analysis of intact molecules without fragmentation. A sample is introduced into a spray chamber at a flow rate of between 0.1-20 μL/min through a stainless steel nebulizer needle. The nebulizer needle has a potential applied to aide in ionization as well as direct the flow of ions into the mass analyzer. The applied voltage produces a charge separation at the surface of the liquid causing the formation of a "Taylor cone" at the tip of the nebulizer needle. The solution in the Taylor cone subsequently reaches a Rayleigh limit, droplets that contain an excess of positive or negative charge detach from the tip and more towards the inlet of the mass spectrometer. The Rayleigh limit is the point at which Coulombic repulsion of the surface charge equals the surface tension of the solution.[88-90] The ESI process is demonstrated in Figure 1.7

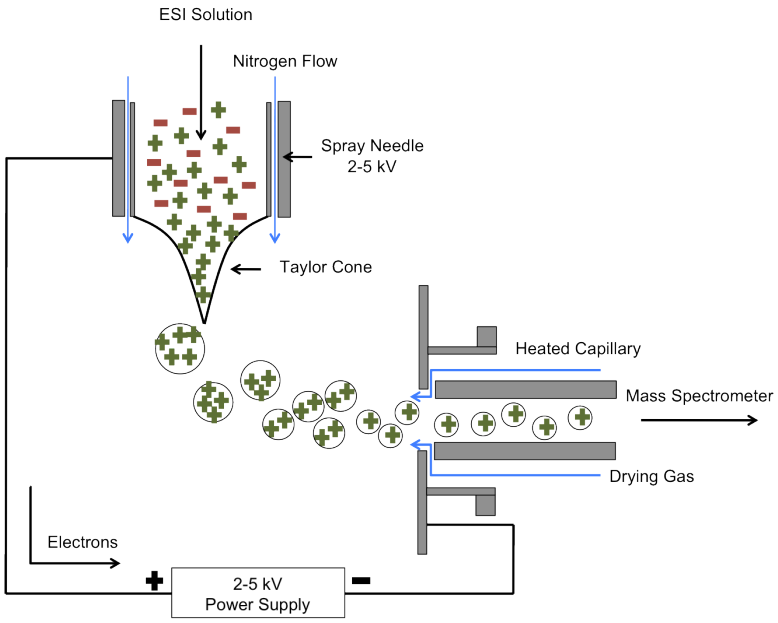


Figure 1.7 Electrospray ionization process description

There are currently two theories of the mechanisms of ion formation after the droplets leave the nebulizer needle. The first is the coulomb fission mechanism, which suggests that the increase charge density due to solvent evaporation causes large droplets to divide into smaller droplets until a single ion is formed.[90] The second mechanism called ion evaporation, suggests that the solvent evaporation causes the increased charge density that eventually causing the coulombic repulsion to become greater than the surface tension of the droplet, resulting in the release of single ions.[90]

ESI can produce neutral ions and also clusters of ions with neutrals that are not introduced into the mass analyzer. To aide in the reduction of these ions, a sheathe gas in utilized to accomplish complete desolvation of the droplets. Another aspect of ESI that reduces the introduction of neutrals into the mass analyzer is the utilization of an orthogonal format between the nebulizer needle and capillary of the mass analyzer.[90]

The mechanism of APCI is not as thoroughly investigated as ESI but APCI experiences a lower susceptibility to matrix effects than ESI. This is due to the fact that APCI takes place in the gas phase.[91] The ionization process in APCI is analogous to chemical ionization. The most commonly used APCI source is corona discharge. In corona discharge, a high voltage is applied to a secondary needle electrode in nitrogen until current in the microampere range are reached.[91] These currents are directly applied to the solvent stream of the sample from a nebulizer needle. The mobile phase evaporates and acts as the ionizing gas to form the reagent ions.[92] A diagram of APCI is shown in Figure 1.8. In the positive ion mode, either proton transfer or adduction of reactant gas ions can occur to produce the ions of molecular species. In negative ionization mode, the ions are produced by either proton abstraction or adduct formation.[92]

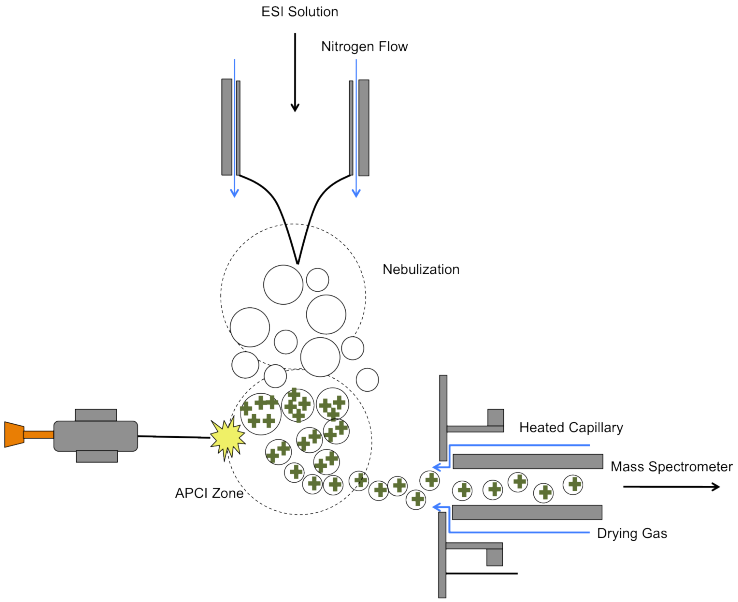


Figure 1.8 Atmospheric Pressure Chemical Ionization depiction

The ionization process in ESI and APCI both occur at atmospheric pressure. Therefore prior to analysis by the mass analyzer, the ions formed must be transferred from

atmospheric pressure to a low-pressure region. The schematic of a TOF-MS is depicted in Figure 1.9.

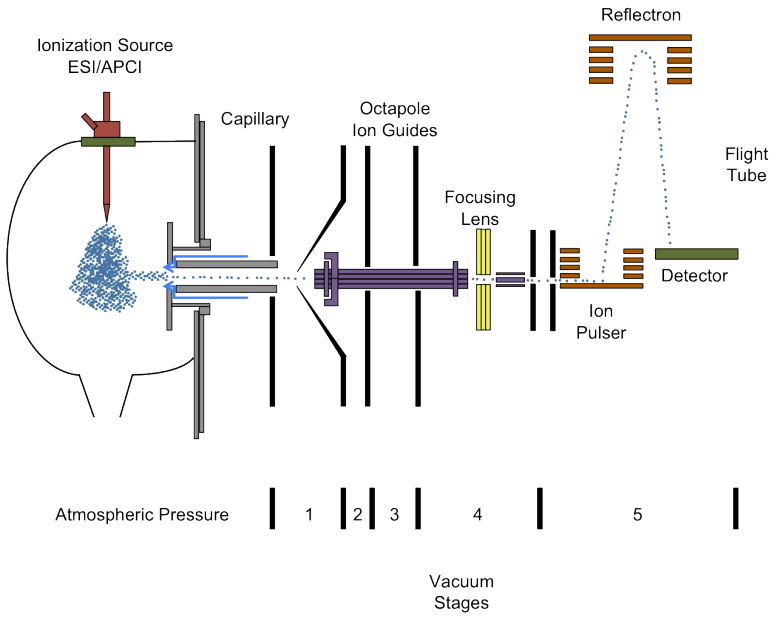


Figure 1.9 Schematic diagram of a TOF-MS

Typically, after ionization the ions are electrostatically drawn through a drying gas and into a heated capillary. The end of the capillary is charged and is a means to introduce the ions into the first stage of the vacuum system. Once the ions pass through the capillary they are introduced to a metal skimmer that has a small aperture to allow ions to pass through while deflecting the air and exhausting it into the rough pump. Once the ions pass through the first skimmer they enter the second stage of the vacuum system.

In the second vacuum state, octopole ion guides have a radio frequency applied to allow ions above a designated mass range to become focused and pass through to the next vacuum stage. In the third vacuum stage the pressure is low enough to cause few collisions between the ions and gas molecules. The ions are introduced to a second

octopole ion guide to accelerate the ions to the fourth vacuum stage, which shapes the ion beam. In the fourth vacuum stage, lenses focus the ions into a parallel beam so that they can be introduced to the time-of-flight (TOF) mass analyzer. The more parallel the ion beam becomes the more resolution in the resulting mass spectrum. Once the ion beam has been formed, the ions pass into the final vacuum stage where the TOF analysis occurs.[93]

The ions pass into a pulser, which starts an orthogonal flight of the ions to the detector. The ions leave the ion pulser and travel through the one-meter flight tube.[93] Two ions of the same mass will leave the pulser at different positions but two ions of the same mass, but different kinetic energies, will leave the pulser at the same time.[94] To minimize kinetic energy variations in arrival time, a reflectron is utilized. This enables ions with a higher kinetic energy to travel further into the reflectron equalizing the arrival time of two ions into the detector.[94] The two ions then travel down the flight tube to the microchannel plate detector. The schematic of the microchannel plate detector is shown in Figure 1.10.

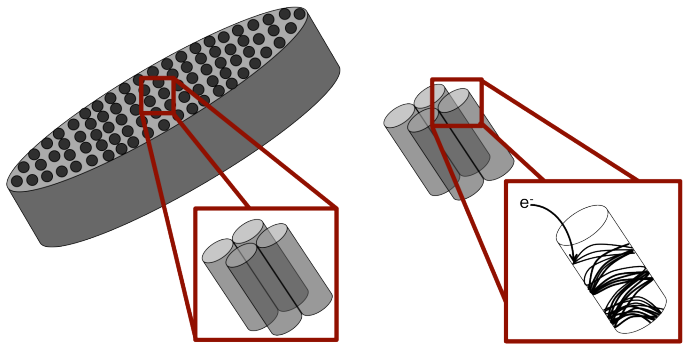


Figure 1.10 Schematic diagram of a microchannel plate detector

The microchannel plate is a very thin plate containing a set of microscopic tubes that pass from the front surface of the plate to the rear surface of the plate.[93] The front surface of

the plate is kept at a negative potential of approximately 1kV compared to the rear surface of the plate.[92] When an ion hits the front of the plate, an electron escapes and begins the process of electrical signal amplification. The channels of the plate are coated with a semiconductor substance enabling secondary electrons to be released. As freed electrons collide with the walls of the tubes, a cascade of electrons travel to the rear of the plate multiplying the number of electrons by a factor of ten.[92]

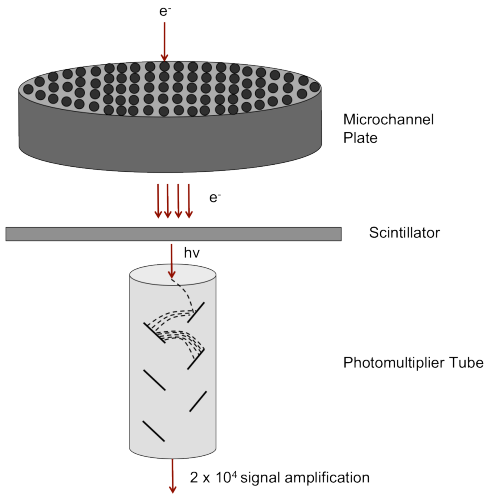


Figure 1.11 Microchannel plate and photomultiplier tube detectors for TOF-MS

Upon leaving the rear side of the microchannel plate, the electrons are focused onto a scintillator as shown in Figure 1.11. A flash of light is produced when the electrons hit the scintillator causing photons to be focused through lenses onto the photomultiplier tube. The photomultiplier tube contains dynodes, which maintain a specific voltage to allow for a cascade of electrons. For each photoelectron that strikes the first dynode, multiple electrons are emitted and are accelerate toward the next dynode. This cascade can produces an amplification of up to 10^6 to 10^7 electrons.[95] The electrical signal produced in the photomultiplier tube is read by the data output system. Therefore, the

overall gain from a single electron entering into the microchannel plate detector to exiting the photomultiplier tube is approximately $2x10^4$.[93]

Chapter 2

Mathematical determination for molecular isotope dilution mass spectrometry

2.1 Traditional IDMS calculations

EPA Method 6800, IDMS, is a quantitative analytical technique based on the measurement of isotopic ratios in a sample that has been spiked with a known amount of an isotopically enriched compound.[42] The relationship between the naturally occurring analyte and the isotopically enriched spike are mathematically expressed using the isotope dilution equation.[42] Traditionally, IDMS quantitation is utilized in the field of elemental speciation and the analysis of metals.[80-83] It is considered a definitive method for trace element analysis and is regarded as a primary ratio method of the highest metrological quality by the International Bureau of Weights and Measures.[84] IDMS has the ability to be applied to the analysis of organic compounds given the availability of different isotopically labeled analogues of the compounds.

Isotope dilution is often times regarded as the only quantitative method systems in which analyte formation occurs during the analysis process.[81] Another commonly stated advantage is that upon equilibration, any loss of analyte is insignificant.[85] It can correct for any matrix effects and partial analyte loss that may occur during sample preparation.[84] Isotope dilution also has the ability to quantitatively assess the transformation between two species that cannot otherwise be determined by traditional quantitative methods.

To ensure unbiased results using IDMS, there are a number of prerequisites that must be addressed when choosing and isotopically enriched analyte. The analyte must be well defined when choosing an isotope for quantitative analysis. The isotope must be stable and enriched at a known purity that also has identical behavior to the analyte. Finally, sampling must be representative between the analyte and spike. Meaning that full equilibration or extraction must occur between the analyte and spike prior to analytical analysis. IDMS techniques and calculations are well established for elemental analysis, but molecular analysis is still in its infancy.[84]

The stability of the isotopically labeled analyte is critical in molecular IDMS. When selecting and isotopically enriched analogue, the isotopically labeled element must be one that cannot be subjected to isotopic exchange. The most well known example of isotopic exchange in a molecular compound is the exchange between hydrogen and deuterium. To avoid isotopic exchange, it is desirable to place the isotopic label in a chemically inert position of the molecule.[84, 96-98] The foundation of IDMS is based on the argument that the chemical and physical behavior between a natural and isotopically enriched analyte is identical. The validity of this premise was investigated by Itoh *et al.* while investigating the difference between ^{2}H and ^{13}C isotopically enriched polycyclic aromatic hydrocarbons (PAH) in sediments. Their results suggest that using ^{2}H-standards produce a consistently lower measured concentration by ~5%. The consistent low bias is explained by the fact that ^{2}H-PAHs have a higher recovery relative to those of ^{13}C-PAH during analytical processes.[99] Although IDMS offers greater precision than standard

addition quantitative methods, the use of some labeled analytes can introduce an inherent slight bias in the isotopic measurement.[99-100]

Although some cases of molecular IDMS have been investigated, it is still in the infancy stages of development. The current IDMS equations are inadequate for accurately calculating the concentration of a molecular species. Currently the contribution of the naturally occurring analyte to the isotopically enriched spike and the contribution of the spike to the analyte are not taken into account. The traditional IDMS equation only takes into account the naturally occurring isotopic abundances for a single element and not for multiple elements. To accurately quantify molecules using IDMS, the probabilities of the contributions of each element in the compound including enriched and non-enriched must be accounted for.

This chapter describes nine different molecules that were utilized for the proper extension of traditional elemental IDMS to molecular IDMS. Glyphosate ($C_3H_8NO_5P$) and methylphosphonic acid ($CH_3P(O)(OH)_2$) are examples of environmental analytes that are also utilized as surrogates for that analysis of nerve agents in water samples. Seven illicit drugs including heroin ($C_{21}H_{23}NO_5$), 6-acetylmorphine ($C_{19}H_{21}NO_4$), morphine ($C_{17}H_{19}NO_3$), cocaine ($C_{17}H_{21}NO_4$), codeine ($C_{18}H_{27}NO$), methadone ($C_{21}H_{27}NO$), and fentanyl ($C_{22}H_{28}N_2O$) were also assessed for quantitation using the developed molecular IDMS equations.

2.2 Determination of IDMS variables for individual compounds

Traditionally, EPA method 6800 is used for elemental analysis of total metals from solid samples or in extracts of digests. Quantitation is based on the addition of a known amount of an isotopically enriched element. Equilibration between the naturally occurring element and isotopically enriched analogue enables a high precision ratio measurement. The traditional IDMS equations is as follows in Equation 2.1:

$$Concentration, \mu g / g = \left(\frac{\left(\left(W_{sp} \cdot C \right) / W_s \right) * \left(A_{sp} - \left(R \cdot B_{sp} \right) \right)}{\left(B \cdot R \right) - A} \right)$$

Where:
W_{sp} = mass of spike solution (g)
C = concentration of spike, µg-atom/g of solution
A_{sp} = atomic fraction of isotope A in spike
B_{sp} = atomic fraction of isotope B in spike
A = atomic fraction of isotope A in sample
B = atomic fraction of isotope B in sample
R = experimentally measured isotope ratio, A/B
W_s = mass of sample, grams

Equation 2.1

When analyzing an element and its isotopically enriched counterpart, B_{sp}, A_{sp}, B, and A are all dependant upon the natural abundances (Table 2.1) of that specific element being analyzed and the purity of the isotopically enriched element. The analysis of a molecular species requires significantly more statistical analysis to determine the constant variables in the IDMS equation. This is due to the fact that not only is there a contribution from the isotopically enriched species to the natural species but also a natural probability of the other atoms in the molecule to contribute both to the naturally occurring peak and isotopically enriched peak in the mass spectra. Another factor that must be assessed is when a molecule is isotopically labeled with two isotopically enriched elements. In this study, glyphosate is an example of a signally enriched atom within a molecule and methylphosphonic acid is an example of double isotope enrichment within a molecule.

Table 2.1. Natural abundances of elements for IDMS quantitation

Element	Natural Abundance	Atomic Mass
Hydrogen	0.999985	1.008
Hydrogen	0.00015	2.014
Carbon	0.98900	12.011
Carbon	0.01100	13.003
Nitrogen	0.99632	14.003
Nitrogen	0.00368	15.000
Oxygen	0.99762	15.995
Oxygen	0.00038	16.999
Oxygen	0.00200	17.999
Phosphorus	1	30.974

The following example is of the determination of the constants B_{sp}, A_{sp}, B, and A for methylphosphonic acid and its doubly labeled isotope methylphosphonic acid-^{13}C-methyl-D_3 shown in Figure 2.1.

Figure 2.1. Chemical structure of methylphosphonic acid and isotopically enriched methylphosphonic acid

To determine A, atomic fraction of isotope A in sample, the purity of the analytical standard of the naturally occurring compound is required. Methylphosphonic acid has a purity of 99% meaning that 99% of the sample in the standard is the most abundant isotopic form (mass 96.02 g/mol). Therefore, the remaining 1% of the standard is the natural isotopic distribution. Equation 2.2 describes the calculation of the probability of having the natural isotopic distribution being non-enriched for methylphosphonic acid.

Equation 2.2 $$probability\ naturally\ non-enriched = \left({}^{12}C\right)\left({}^{1}H\right)^{5}\left({}^{16}O\right)^{3}\left({}^{31}P\right)$$

$$0.98 = (0.98900)(0.99985)^{5}(0.99762)^{3}(1)$$

$$A = (99\%) + (1\%)(0.98)$$

Where the values for ${}^{12}C$, ${}^{1}H$, ${}^{16}O$, and ${}^{31}P$ are the natural abundances of each element in the lowest isotopic form. The powers represent the number of each atom in the compound.

Constant, *B*, is the atomic fraction of isotope B in the sample. This value is obtained from the 1% of standard methylphosphonic acid that is naturally isotopically enriched to obtain a four Dalton peak up-shift in the mass spectra (mass 100.02 g/mol). This value is estimated to be 0.00000001 due to the low probability of the 1% of the sample containing any methylphosphonic acid that is naturally labeled to create a four Dalton peak up-shift.

To determine A_{sp}, atomic fraction of isotope A in spike, the probability of the spike sample contributing to the most predominate naturally occurring mass must be determined. Equation 2.3 depicts calculating A_{sp} for methylphosphonic acid.

Equation 2.3 $$probability\ enriched\ contributing\ to\ natural = \left({}^{12}C\right)\left({}^{1}H\right)^{3}\left({}^{1}H\right)^{2}\left({}^{16}O\right)^{3}\left({}^{31}P\right)$$

$$7.939x10^{-8} = (0.01)(0.02)^{3}(0.99985)^{3}(0.99762)^{3}(1)$$

Where both ${}^{12}C$ and $({}^{1}H)^{3}$ are the probabilities of the isotopically enriched standard to be naturally labeled. These values are given from the purities of the isotopically enriched standard. Methylphosphonic acid-^{13}C-methyl-D_3 has a ${}^{13}C$ purity of 99% and a methyl-D_3 purity of 98%. Therefore, the probability of ${}^{12}C$ is 0.01 and methyl-D_3 is 0.02.

To calculate, B_{sp}, the probabilities of the spike having a natural four Dalton mass up-shift along with the probability of the most abundant isotopic form in the standard must be calculated. The purity of the isotopically enriched standard determines the contribution of the naturally occurring abundance in the spike having a four Dalton up shift. Equation 2.4 describes the general format for the calculation of B_{sp} for methylphosphonic acid.

Equation 2.4

$$B_{sp} = \left(probability\,of\ most\,abundant\right) + (1\%)\left(probability\,of\ permutations\right) + (2\%)\left(probability\,of\ permutations\right)$$

The probability of the most abundant isotopic form in the spiked sample is calculated using Equation 2.5.

Equation 2.5

$$probability\,of\ most\,abundant = \left({}^{13}C\right)\left({}^{2}H\right)\left({}^{1}H\right)^{2}\left({}^{16}O\right)^{3}\left({}^{31}P\right)$$

$$probability\,of\ most\,abundant = (0.99)(0.98)(0.9999)^{2}(0.998)^{3}(1)$$

There are 204 permeations of atoms in methylphosphonic acid that will give a four Dalton mass up-shift. The additive probabilities equates to $1.3x10^{-5}$. This value is then multiplied by the percentage of impurity in the isotopically enriched standard. The additive value of all portions of the equations equate to B_{sp}. This computation is depicted in Equation 2.6.

Equation 2.6

$$B_{sp} = (0.9642) + (1\%)\left(1.3x10^{-5}\right) + (2\%)\left(1.3x10^{-5}\right)$$

$$B_{sp} = 0.9642$$

To properly utilize EPA method 6800 for the analysis of organic compounds, the natural abundance of all atoms in the compound along with the purity of the isotopically enriched element must be statistically determined and accounted for. The statistical determination was completed for the constants B_{sp}, A_{sp}, B, and A in the IDMS calculation. Constant values were determined for glyphosate, methylphosphonic acid, heroin, 6-

acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl. These constant values are shown in Table 2.2. The isotopically enriched analogues for each analyte were glyphosate-2-^{13}C, methylphosphonic acid-^{13}C-methyl-D_3, heroin-D_9, 6-acetylmorphine-D_3, morphine-D_3, cocaine-D_3, codeine-D_3, methadone-D_3, and fentanyl-D_5.

Table 2.2 Statistical determination of analyte constants for molecular IDMS

Analyte	B_{sp}	A_{sp}	B	A
Glyphosate	0.9527	0.00951	0.9512	0.04768
Methylphosphonic acid	0.9642	7.936×10^{-8}	1.0×10^{-8}	0.9997
Heroin	0.6493	3.988×10^{-16}	1.0×10^{-8}	0.0152
6-Acetylmorphine	0.7508	6.381×10^{-6}	1.0×10^{-8}	0.0156
Morphine	0.7696	6.542×10^{-6}	1.0×10^{-8}	0.0160
Cocaine	0.7675	6.524×10^{-6}	1.0×10^{-8}	0.0160
Codeine	0.7606	6.468×10^{-6}	1.0×10^{-8}	0.0158
Methadone	0.7389	6.281×10^{-6}	1.0×10^{-8}	0.0184
Fentanyl	0.7282	2.476×10^{-9}	1.0×10^{-8}	0.0152

With the determination of these constants for the molecules being analyzed, the IDMS equation can be extended to the analysis of complex molecules and is not limited to the analysis of elemental species. With the utilization of these constants, the IDMS equation now takes into account contributions of standard purity, analyte to spike contributions, and spike to analyte contributions. The knowledge of these probability equations has the potential to become a mathematical program to determine the constants B_{sp}, A_{sp}, B, and A for any molecular compound.

2.3 Conclusions

Traditional IDMS equations were modified for the accurate quantitation of molecular compounds. Traditional IDMS equations are utilized for elemental analysis taking into account only the natural abundances of the element itself. When assessing molecules for quantitation, the elements other than the isotopically enriched element also contribute to

the naturally occurring probability of a contribution of analyte to spike and spike to analyte. Therefore, statistical analysis needs to be completed for the probabilities of B_{sp}, A_{sp}, B, and A for any molecular compound. Table 2.2 illustrates the IDMS constants for glyphosate, methylphosphonic acid, heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl.

The isotopic enrichment directly contributes the constants B_{sp}, A_{sp}, B, and A for any molecular compound. Glyphosate is an example of a compound that has a single label on a carbon atom within the molecule. This single label causes only a one Dalton up shift in the mass spectra for glyphosate-2-^{13}C compared to the analyte. Therefore, there is a greater probability of a contribution of other natural abundance from other elements in the compound such as oxygen, hydrogen, nitrogen, and carbon. The more isotopic enrichments in a given molecule, the lower the probability that the analyte will naturally contribute to the spike peak. This is seen in A_{sp}, where glyphosate has a single enrichment and a value of 0.00951 and methylphosphonic acid, with four enrichments, has a value of 7.936×10^{-8}. This is also seen in the illicit drug analysis with deuterium labeled analytes. Heroin has nine deuterated hydrogen atoms and an A_{sp} value of 3.988×10^{-16} while 6-acetylmorphine has only three deuterated atoms hydrogen and an A_{sp} value of 6.381×10^{-6}.

With the determination of these constants for the molecules being analyzed, the IDMS equation can be extended to the analysis of complex molecules and is not limited to the analysis of elemental species. With the utilization of these constants, the IDMS equation now takes into account contributions of standard purity, analyte to spike contributions,

and spike to analyte contributions. The contribution determination is important in the analysis of molecules when using IDMS because of the availability of carbon-13 labeled standards. A single Dalton up shift has the most significant values for the IDMS equation. These equations have been utilized in the subsequent chapters of this dissertation.

Chapter 3

Quantitation of phosphate based environmental molecules in water samples

3.1 Introduction

Glyphosate, a nonselective post-emergent herbicide, is extensively used in the United States for total vegetation control.[2-4] It is the active ingredient in the commercially available herbicide, Roundup®, which has been extensively used in the United States since its introduction in 1974.[7-8] Although the toxicity is low, accidental death by ingestion of large amounts of Roundup has increased since 1987.[7-8] The current maximum contaminant level goal designated by the United States Environmental Protection Agency (USEPA) for glyphosate in drinking water is 0.7 ppm.[11] The analysis of glyphosate via GC-MS and HPLC requires tedious and time-consuming derivatizations due to polar and ionic character of the analytes.[1-2, 8, 18, 101-103] Recently, methods have been developed for the analysis of glyphosate using liquid chromatography-mass spectrometry (LC-MS). However, they retain the required derivatization step for column retention.[2-3, 24, 104-106]

SPE with an anion exchange resin utilizing the ionic character of glyphosate was used for sample clean up and concentration. Two SPE methods were developed for the analysis of glyphosate in drinking water by following the EPA Method 6800, IDMS, for quantitation.[107] IDMS is a quantitative analytical technique based on the measurement of isotopic ratios in a sample that has been spiked with a known amount of an isotopically

enriched compound. The employment of IDMS in conjunction with SPE eliminates concerns of incomplete recovery and elution, along with potential adverse effects of matrix and sorbent on ionization and mass spectrometry performance. Once equilibration is achieved, any chemical or physical change occurs equally between the species of interest, therefore ionization and most mass spectral errors are reduced or eliminated in significance and accuracy is increased.

This chapter describes two novel methods for the analysis of glyphosate in drinking water via ESI-TOF-MS that does not involve tedious and time-consuming derivatization. SPE-IDMS and i-Spike were validated for the quantitation of glyphosate in drinking water. SPE-IDMS involves pre-equilibration of the sample prior to SPE while i-Spike facilitates the addition of the isotopically enriched analyte onto the SPE column prior to the addition of the sample containing analyte. After the analyte containing sample is loaded onto the SPE column, both the analyte and spike are co-eluted from the solid phase. The eluate is then directly analyzed by mass spectrometry. i-Spike is advantageous since it enables the isotope to be preloaded onto the column prior to the addition of the analyte, enabling a rapid, simple and low cost field analysis technique. i-Spike has the potential to be useful for applications in forensics, homeland defense and environmental health assessment areas with future automation for high-throughput applications.

3.2 Materials and methods

3.2.1 Reagents and supplies

Glyphosate (99%) analytical standard and glyphosate-2-^{13}C (^{13}C, 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water, acetonitrile, and

methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Bioanalytical grade formic acid was also purchased from Fisher Scientific. The Strata-SAX (500 mg bed mass, 6 mL volume capacity) solid phase extraction columns were purchased from Phenomenex (Torrance, CA, USA). Drinking water was supplied from Pittsburgh municipal water supply (Pittsburgh, PA, USA).

3.2.2 Instrumentation

3.2.2.1 ESI-TOF-MS

Both Agilent Technologies 6210 series TOF (Santa Clara, CA, USA) and Bruker Daltonics microTOF (Billerica, MA, USA) mass spectrometers with orthogonal ESI sources equipped with respective data analysis software were optimized for the analysis of glyphosate. Analyses were implemented with direct infusion into the ESI source at a flow rate of 240 μL/hour with a Cole Palmer 74900-00 syringe pump (Vernon Hills, IL, USA). All analyses were completed in negative ionization mode with a mass to charge range of 50-1000 *m/z*. Initial method development was completed on an Agilent Technologies TOF with the following operating conditions: capillary voltage 2500 V; gas temperature 275 °C; drying gas 7.0 L/min; nebulizer 12 psig; fragmentor 150 V; skimmer 60 V; oct RF voltage 250 V. The optimized Bruker microTOF operating conditions for the analysis of glyphosate are: capillary voltage +3750 V; nebulizer pressure 0.5 Bar; nitrogen drying gas temperature 200 °C; capillary exit -100 V; skimmer 1 -40.0 V; hexapole 1 -23.0 V; hexapole RF 65 Vpp; skimmer 2 -22.0 V. Samples were collected in four replicate samples for a time of 5 minutes per replicate for statistical analysis. The peak area and peak abundance for naturally occurring glyphosate (168.0 *m/z*) and

isotopically enriched glyphosate-2-^{13}C (169.0 *m/z*) were recorded for each sample for quantitation using IDMS.

3.2.2.2 APCI-Q-TOF-MS

The Agilent Technologies 6530 Auccurate-Mass quadrupole-TOF LC/MS (Santa Clara, CA, USA) was optimized for the analysis of glyphosate. Analyses were implemented with direct infusion into the APCI source at a flow rate of 0.5 mL/min with a Cole Palmer 74900-00 syringe pump (Vernon Hills, IL, USA). All analyses were completed in negative ionization mode with a mass to charge range of 50-200 *m/z*. The optimized APCI-Q-TOF-MS settings are: gas temperature 300 ^{o}C; vaporizer 350 ^{o}C; nitrogen drying gas 6 L/min; nebulizer 35 psig; capillary voltage 4500 V; corona 18 μA; fragmentor 115 V; skimmer 65 V; oct 1 rf vpp 110 V. Samples were collected in four replicate samples for a time of 1 minute per replicate for statistical analysis. The peak area and peak abundance for naturally occurring glyphosate (168.0 *m/z*) and isotopically enriched glyphosate-2-^{13}C (169.0 *m/z*) were recorded for each sample for quantitation using IDMS.

3.2.3 Dynamic range analysis

The calculation of the error propagation factor for each analyte determines the ideal ratio between the naturally occurring analyte and the isotopically enriched analyte.

Equation 3.1 expresses the equation of determining the error propagation factor (EPF) for a given naturally occurring analyte and isotopically enriched isotope.

Equation 3.1. $$EPF = \sqrt{\frac{isotope\ A\ spike\ abundance}{isotope\ B\ spike\ abundance} x \frac{isotope\ A\ natural\ abundance}{isotope\ B\ natural\ abundance}}$$

The optimal spiking ratio for glyphosate was calculated to be 1.0 when using

Equation 3.1. Theoretically a spike ratio does not have to precisely be the optimal ratio determined by the EPF. Deviation from the optimal ratio will give an increased error at a determined point. Therefore, a dynamic range analysis was completed for glyphosate in water.

Samples of glyphosate were prepared at 1:10, 1:2, 1:1, 2:1, and 10:1 ratio of naturally occurring analyte and isotopically enriched spike. The samples were prepared by mass and then analyzed on an ESI-TOF-MS, atmospheric pressure chemical ionization-quadrupole-mass spectrometer (APCI-QQQ-MS), and atmospheric pressure chemical ionization-quadrupole-time of flight-mass spectrometer (APCI-Q-TOF-MS) and compared for quantitative significance. The peak abundances for both the analyte and spike *m/z* peaks were recorded for IDMS quantitation.

3.2.4 Solid phase extraction method development/validation

Extractions were performed on a Strata-SAX solid phase extraction column with a 500 mg bed mass and a 6.0 mL column volume. SPE-IDMS and i-Spike extraction methods were developed for the analysis of glyphosate in drinking water samples. The SPE analyses were completed with a flow rate of 1.0 mL/min maintained by a negative pressure SPE vacuum chamber (Supelco, St. Louis, MO). Comparison analysis between traditional IDMS, SPE-IDMS, and i-Spike were performed with HPLC grade water spiked with both naturally occurring analytes and their isotopically enriched spikes. Comparison analysis was also completed in drinking water samples with SPE-IDMS and i-Spike. Column limit of quantitation and sample concentration were determined in

drinking water samples. Initial method development was completed on an ESI-TOF-MS and then extended to an APCI-Q-TOF-MS.

Assessing the pH of the analyte as well as the elution solvent system and volume developed the extraction method. To assess the elution solvent system, a 2:1 acetonitrile:methanol, 1:2 acetonitrile:methanol, and 1:1 acetonitrile:methanol system all with 6% formic acid were assessed. The eluate was collected in 2.0 mL fractions for a total elution volume of 14.0 mL. The eluate fractions as well as the flow through from the wash and load steps were analyzed using ESI-TOF-MS. The 168.0 *m/z* peak was analyzed for intensity and was plotted versus fraction number to determine the optimal solvent system for elution glyphosate from the Strata-SAX column. The determination of the optimal elution volume was also determined from the intensity vs. fraction number plot of the optimal solvent system. The glyphosate must be fully eluted from the SPE column for analysis and therefore determining the elution volume from the intensity vs. fraction number plot of the optimal solvent system. The final assessment made was the analysis of the optimal pH for glyphosate when it is loaded onto the SPE column. This was determined by loading glyphosate onto the SPE column at pH values 4, 6, 9, and 10. After washing the column, the eluate was collected in 2.0 mL fractions up to a final eluate volume of 18.0 mL. The fractions were then analyzed on an ESI-TOF-MS assessing both 168.0 *m/z* and 169.0 *m/z* for peak intensity. The peak intensities were plotted against the fraction number to assess the pH and confirm the optimal elution volume.

3.2.4.1 SPE-IDMS

Water samples were spiked by mass at a 1:1 ratio with naturally occurring glyphosate and isotopically enriched glyphosate-2-^{13}C at equivalent concentrations. The Strata-SAX SPE column was conditioned with 4.0 mL of HPLC grade methanol. The column was then equilibrated with 4.0 mL of HPLC grade water. Four grams of equilibrated water sample was then loaded and the column was then washed with 4.0 mL of HPLC grade methanol prior to elution with 16.0 mL of 6.0% formic acid in 1:1 acetonitrile:methanol solution. The eluate was analyzed by ESI-TOF-MS and APCI-Q-TOF-MS. The method was validated by assessing the limit of quantitation, SPE column limit of quantitation, and limit of concentration of analyte onto the SPE column.

To determine the instrument limit of quantitation, water samples were prepared that were spike at a 1:1 ratio of glyphosate and glyphosate-2-^{13}C at decreasing concentrations. The samples were then analyzed via ESI-TOF-MS and APCI-Q-TOF-MS until the signal to noise ratio was less than 3:1 indicating the limit of detection. The same data was utilized to determine the limit of quantitation. The peak abundances of both analyte and isotope were utilized to quantify the analyte by IDMS.

To determine the limit of quantitation of the SPE column, drinking water samples were spiked with both the naturally occurring analyte and isotopically enriched spike. Samples were prepared in decreasing concentration and subjected to the SPE procedure. After SPE, the samples were analyzed to determine the lowest quantifiable concentration.

The analytes were also concentrated onto the SPE column to further decrease the limit of quantitation. Samples were spiked at a 1:1 ratio at varying concentrations making sure that enough quantity of sample was prepared to concentrate the sample up to a concentration of 6.45 ppm. After SPE, the samples were analyzed by both ESI-TOF-MS and APCI-Q-TOF-MS.

3.2.4.2 i-Spike

Loading the naturally occurring analyte and isotopically enriched isotope by mass individually onto the SPE column completed the method development for the i-Spike method. The Strata-SAX column was conditioned with 4.0 mL of HPLC grade methanol and equilibrated with 4.0 mL of HPLC grade water prior to loading the samples. Four grams of isotopically enriched spike was then loaded onto the column followed by loading 4.0 g of naturally occurring analyte at equivalent concentrations. The samples were quantitatively transferred onto the column by washing the container and pipette tip used with three 1.0 mL volumes of HPLC water. The SPE column was then washed with 4.0 mL of HPLC grade methanol followed by elution with 16.0 mL of 6.0% formic acid in a 1:1 acetonitrile:methanol solution. The eluate was analyzed by both ESI-TOF-MS and APCI-Q-TOF-MS. Method validation was completed by determining the limit of quantitation, SPE column limit of quantitation, and limit of concentration of sample onto the SPE column.

To determine the limit of quantitation of the SPE column, drinking water samples were spiked with both the naturally occurring analyte and isotopically enriched analyte.

Samples were prepared in decreasing concentration and subjected to the SPE procedure. After SPE, the samples were analyzed to determine the lowest quantifiable concentration.

The analytes were also concentrated onto the SPE column to further decrease the limit of quantitation. Samples were spiked at a 1:1 ratio at varying concentrations making sure that enough quantity of sample was prepared to concentrate the sample up to a concentration of 6.45 ppm. After SPE, the samples were analyzed by both ESI-TOF-MS and APCI-Q-TOF-MS.

3.2.5 i-Spike time stability analysis

The stability of the isotopically enriched spike on the SPE column is essential for field analysis/commercialization of the i-Spike method. To determine the viability of the isotope on the SPE column, various methods were assessed to optimize the isotopes elution from the column. The first method involved loading 4.0 mL of isotope onto the top of the SPE column and enabling the column to air dry. The columns dried for one week, two weeks, and one month prior to loading the analyte onto the column followed by elution. Four SPE columns were prepared for each time point for statistical analysis. The next methods involved keeping the SPE column wet with either 2.0 mL of methanol or 2.0 mL of HPLC grade water. The addition of water/methanol was after loading the isotope onto the SPE column. Four columns were prepared for each solvent and were incubated for one week prior to the addition of the analyte and subsequent elution and quantitation.

A reverse time stability study was performed to determine if irreversible binding or tunneling was occurring in the SPE column after the addition of the isotope. Four SPE columns were loaded with 4.0 mL of analyte and were incubated for one week prior to loading the isotope. The eluate was analyzed and IDMS was performed to determine stability.

To increase the stability of the isotope on the SPE column, 0.22 mL of concentrated isotope was loaded onto the SPE column and enabled to air dry for a period of one week and two weeks. Four SPE columns were used for each time point in the analysis for statistical comparison. After the designated drying time, the 4.0 mL analyte was loaded onto the column at an equivalent concentration to the 0.22 mL of isotope.

A final method to increase the stability was to introduce a secondary frit to the SPE column. The frit was place in cartridge approximately 0.5 cm above the existing frit on the column packing. After placing the frit above the column, 0.22 mL of concentrated isotope was placed on the frit and allowed to air dry for a time period of one week, two weeks, and one month. Four columns were assessed for each time point for statistical analysis. After the designated drying time, 4.0 mL analyte was loaded onto the column at an equivalent concentration to the 0.22 mL of isotope. All samples were analyzed on the ESI-TOF-MS and quantified by IDMS.

3.3 Results and discussion

3.3.1 Instrumental method optimization

The quantitative analysis of glyphosate in water was optimized using both an ESI-TOF-MS and an APCI-Q-TOF-MS with the parameters described in Section 3.2.2.1 and Section 3.2.2.2. Figure 3.1 depicts the ESI-TOF-MS analysis of glyphosate (red), glyphosate-2-^{13}C (green), and a 1:1 ratio of glyphosate:glyphosate-2-^{13}C (blue). The axes are offset by 0.5 *m/z* and 100 counts in the *x* and *y* directions.

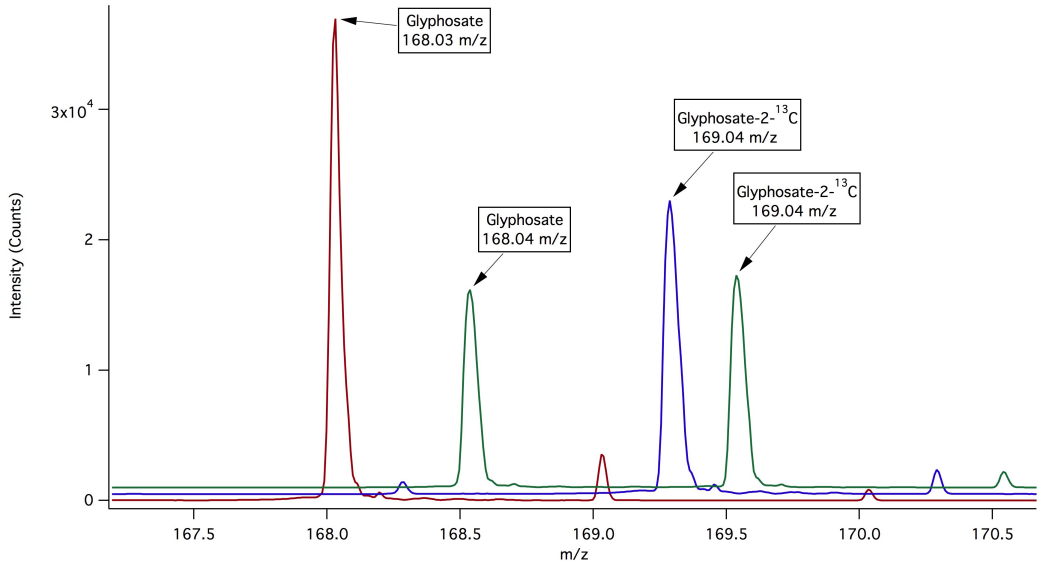


Figure 3.1. ESI-TOF-MS analysis of glyphosate standard and isotopically enriched glyphosate

3.3.2 Dynamic range analysis

The optimal analyte to spike ratio was determined to be 1:1 for glyphosate after calculation of the error propagation factor. To determine the dynamic range of the ratio between the analyte and isotope, a variety of ratios were analyzed. Upon analysis and IDMS quantitation of the dynamic range samples for the ESI-TOF-MS it was determined that signal suppression was occurring. When analyzing a sample that had a higher portion of analyte to isotope, the isotope signal was always suppressed. This was also seen in the

opposite direction. In cases where the isotope concentration was higher than the analyte, the analyte signal was always suppressed. To determine if the phenomenon was indeed ion suppression in the ionization source, samples were analyzed using the APCI-QQQ-MS. Samples were analyzed in both Scan and SIM modes and quantified by IDMS. The utilization of the APCI source provides a constant ionization source from the corona discharge needle. In ESI, there are a limited number of ions that can be formed in the ionization process unlike the APCI constant ionization source.

The unit mass resolution in the APCI-QQQ-MS is a drawback for the analysis of glyphosate and isotopically enriched glyphosate due to the one mass unit differential. In QQQ-MS, the analyte and isotope peak are not fully resolved causing slight error in the quantitation of the analyte. Therefore, the APCI source was utilized on a Q-TOF-MS creating the optimal instrument for the analysis of glyphosate. The constant ionization from the corona discharge along with the resolution of the Q-TOF-MS enables precise and accurate quantitation of glyphosate at ratios of 1:10, 1:2, 1:1, 2:1, and 10:1. This means that the analysis of a drinking water sample of an unknown concentration of glyphosate can accurately and precisely be quantified at ratios as extreme as 1:10 and 10:1. This is also dependant on the background of the APCI-Q-TOF-MS being utilized. If a higher background level is seen in the instrument, a ratio of 1:2 or 2:1 may be preferred as to not make the lower proportioned molecule below the limit of detection.

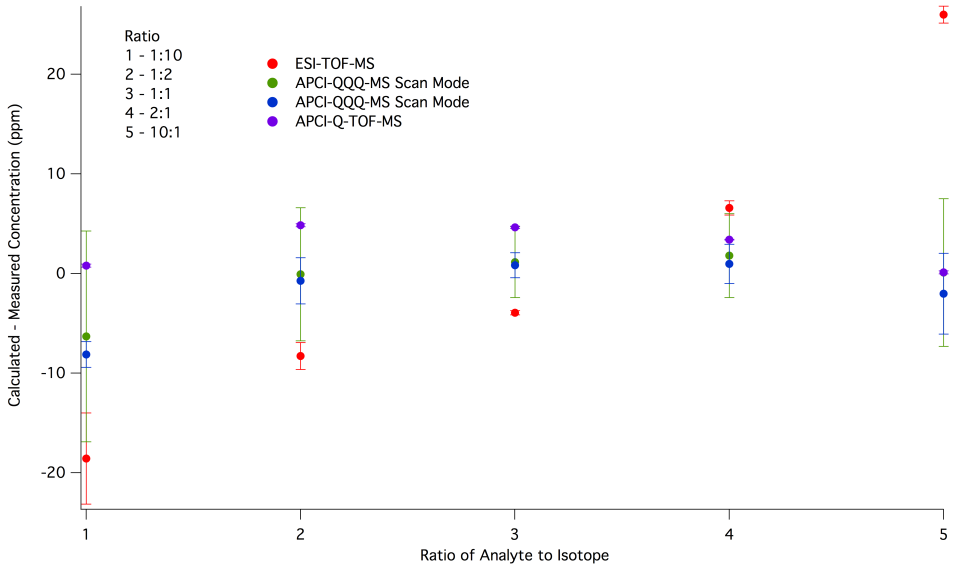


Figure 3.2. Dynamic range analysis of glyphosate utilizing ESI and APCI ionization sources

Figure 3.2 compares the dynamic range analysis of glyphosate utilizing both ESI and APCI ionization sources. ESI-TOF-MS quantitation is demonstrated in red indicating an increased error in the measurement, as the ratio of analyte to isotope is increased. The calculated concentration of glyphosate was 52.000 ppm. At a 1:1 ratio, the measured concentration was 55.931±0.21 ppm (n=4). As the ratio of analyte to isotope increased to 1:10 ratio the measured concentration increased to 70.572±4.57 ppm (n=4). When the ratio of analyte to isotope decreased to 10:1 the measured concentration decreased to 26.005±0.84 ppm (n=4). This is indicative of ion suppression within the ESI ionization source. APCI-QQQ-MS was analyzed in both Scan (green) and SIM (blue) mode indicating a decreased error and more accuracy within the measurement as compared to ESI-TOF-MS. The analysis was then extended to an APCI-Q-TOF-MS (purple). The measured concentration of a 1:1 glyphosate sample from the APCI-Q-TOF-MS was 47.358±0.11 ppm (n=4). As the ratio deviated to ratios of 1:10 to 10:1 the measured concentrations were 51.198±0.16 and 51.887±0.16 ppm (n=4) respectively. The corona

needle in the APCI source enables a constant source of ions, which reduces the signal suppression from an analyte or isotope. APCI-Q-TOF-MS is the optimal instrument configuration for the analysis of glyphosate that does not produce ion suppression and has high resolution.

3.3.3 SPE method development/validation

A SPE method for the analysis of glyphosate in water samples was developed by optimizing the elution solvent system and volume as well as the pH of the analyte upon loading onto the SPE column. Figure 3.3 represents the optimized solvent system for the elution of glyphosate from the SPE column. The optimized solvent system is 6% formic acid in and an acetonitrile/methanol solution. The solvent system enabled glyphosate to be eluted from the SPE column in a more efficient manner than a 2:1 and 1:2 ratio of acetonitrile:methanol.

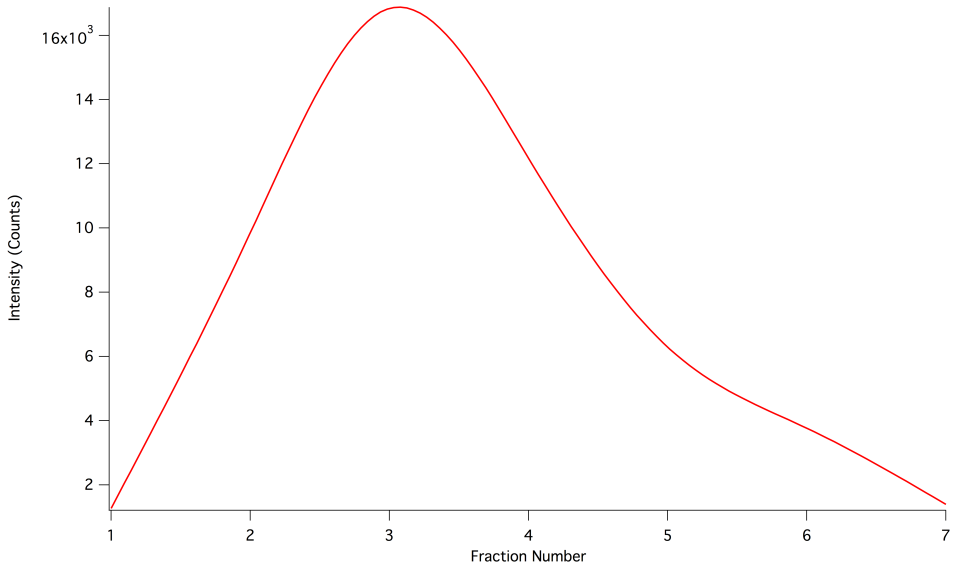


Figure 3.3. Elution solvent system evaluation for the solid phase extraction of glyphosate.

The optimal loading pH for glyphosate onto the SPE column was determined by varying the pH of the analyte as it was loaded onto the column. The pH values analyzed were 4,

6, 9, and 10. After loading the glyphosate onto the column the optimized elution solvent system was utilized to elute the analyte in 2.0 mL fractions that were subsequently analyzed by ESI-TOF-MS. Figure 3.4 shows the fraction analysis of the optimal pH value of 6.0 for glyphosate (blue) and glyphosate-2-^{13}C (red). The analyte and isotope are eluted from the SPE column simultaneously making total elution volume of 16.0 mL. This volume enables both analyte and isotope to be fully eluted from the SPE column for accurate quantitation by IDMS.

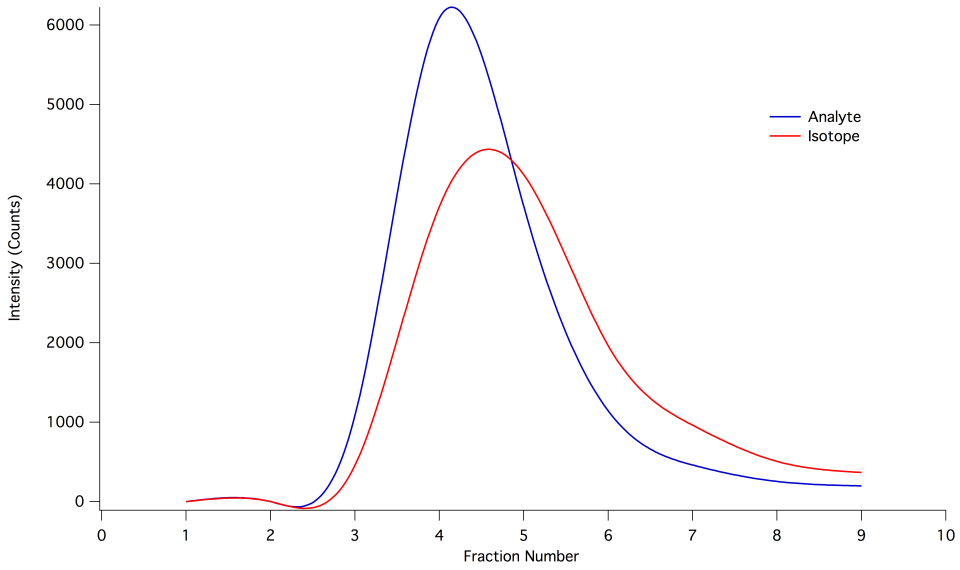


Figure 3.4. Determination of optimal pH of glyphosate loaded onto SPE column

To validate the newly developed solid phase extraction procedures IDMS, SPE-IDMS, and i-Spike methodologies were compared using both ESI-TOF-MS and APCI-Q-TOF-MS. Figure 3.5 demonstrates the similarities in the quantitation of glyphosate using the three sample preparation methods prior to ESI-TOF-MS analysis. All three methods were normalized to a concentration of 100.00 ppm. Traditional IDMS had a measured concentration of 98.627±2.96 ppm (n=32). The SPE-IDMS and i-Spike sample

preparation methods had a measured concentration of 98.625±2.95 ppm (n=20) and 100.297±2.16 ppm (n=20) respectively.

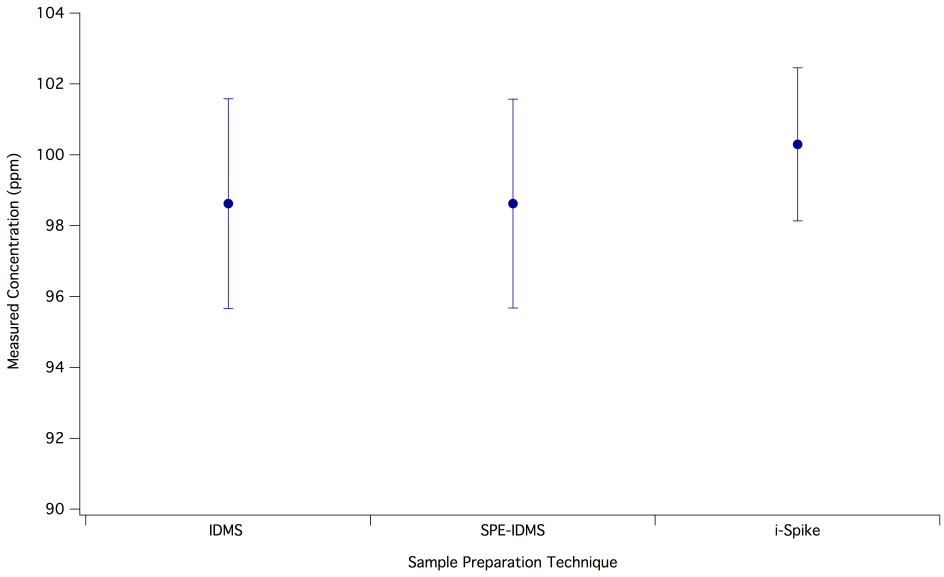


Figure 3.5. Comparison of IDMS, SPE-IDMS, and i-Spike using ESI-TOF-MS

Figure 3.6 demonstrates the similarities in the quantitation of glyphosate using the three sample preparation methods prior to APCI-Q-TOF-MS analysis. The normalized calculated concentration was 6.00 ppm for all three methods. Traditional IDMS had a measured concentration of 6.246±0.081 ppm (n=32). The SPE-IDMS and i-Spike sample preparation methods had measured concentrations of 6.069±0.016 (n=20) and 5.925±0.052 ppm (n=20) respectively. The measured concentrations for ESI-TOF-MS and APCI-Q-TOF-MS were well within the USEPA accepted 20% error measurement.

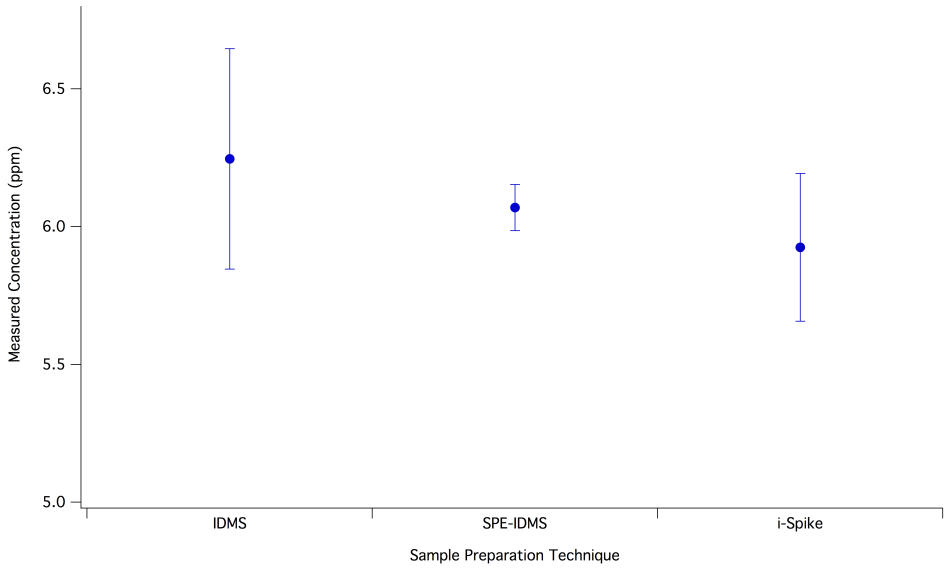


Figure 3.6. Comparison of IDMS, SPE-IDMS, and i-Spike using APCI-Q-TOF-MS

To further validate the SPE methods, column quantification limits were determined using ESI-TOF-MS as shown in Figure 3.7. For the analysis of glyphosate in drinking water, the limit of quantitation with the SPE column is 0.97±0.05 ppm (n=4) for both SPE-IDMS (red) and i-Spike (blue). This is due to volumetric difference between the eluate of the SPE process and the loading of samples onto the column was 4.0 mL. This dilution of the sample in the SPE process causes the limit of quantitation to be higher than the instrumental limit of quantitation that was determined to be as low as 0.312 ppm for ESI-TOF-MS analysis and 1.5 ppm for APCI-Q-TOF-MS analysis. Therefore, glyphosate samples must be concentrated onto the SPE column to meet current drinking water regulations. The maximum containment levels developed by National Primary Drinking Water Regulations from the USEPA is 0.700 ppm.

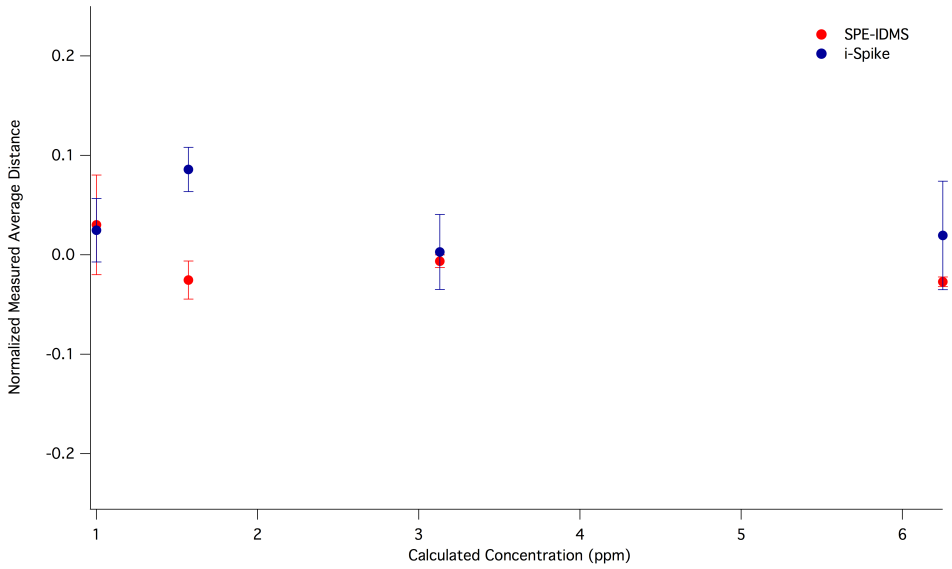


Figure 3.7. SPE-IDMS and i-Spike limit of quantification determination

Decreasing the current limit of quantitation of the SPE column to reach USEPA standards required concentration of the analyte onto the SPE column. Analyte concentration was assessed for both SPE-IDMS and i-Spike methodologies as described in Figure 3.8. With decreased concentration, larger masses of spike glyphosate samples were loaded onto the column to concentrate glyphosate up to a concentration of 6.0 ppm. The employment of concentrating glyphosate on the SPE-IDMS column decreases the limit of quantitation of the SPE column from 0.97±0.05 ppm (n=4) to 0.40 ppm concentrated onto the column up to a measured concentration of 5.95±0.08 ppm (n=4).

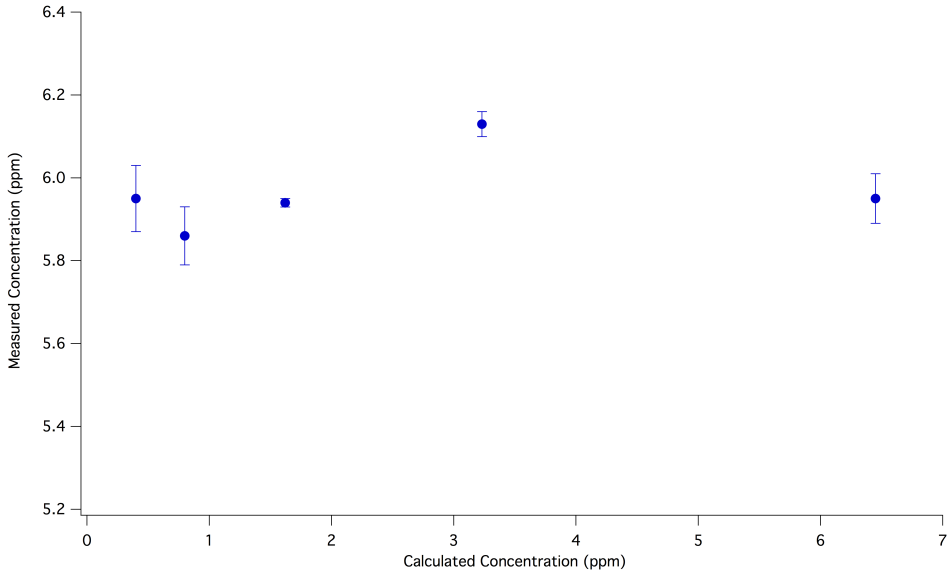


Figure 3.8. Concentration of glyphosate using SPE-IDMS

Concentration of glyphosate onto the SPE column using the i-Spike method was carried out in two different manners to significantly decrease quantitation limit values. First equivalent masses of naturally occurring glyphosate standard and isotopically enriched glyphosate-2-^{13}C were loaded onto the SPE column. With decreased sample concentration, an increased sample mass was loaded onto the column to concentrate the sample up to 6.25 ppm. As described in Figure 3.9, loading equivalent masses of analyte and spike onto the column and concentrating glyphosate up to a concentration of 6.25 ppm does not significantly decrease the column limit of quantitation due to overloading of the SPE column. Therefore, a second sample concentration was completed by maintaining a constant isotopically enriched glyphosate2-^{13}C loading at 4.0 g of a 6.25 ppm samples and increasing the naturally occurring glyphosate sample size with decreased concentration. This dramatically decreased the limit of quantitation for the column from 0.97±0.03 ppm (n=8) to 0.40±0.01 ppm (n=4).

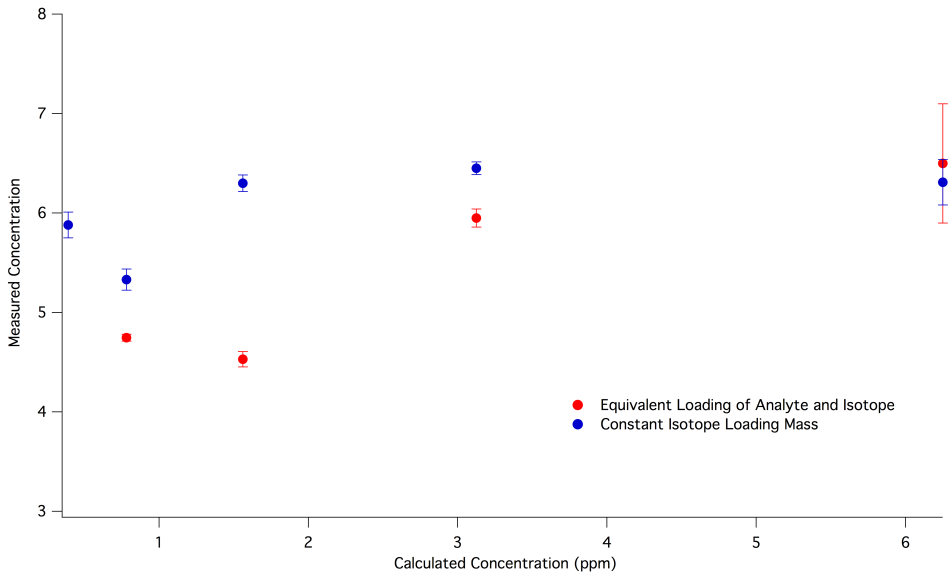


Figure 3.9. Concentration of glyphosate using i-Spike

3.3.1 i-Spike time stability analysis

The stability of the isotopically enriched glyphosate on the SPE column was determined to establish the capacity of i-Spike becoming a field portable sample preparation method. The stability of the isotope is essential in determining prepackaging time limitations prior to field analysis. Four different methods were assessed to extend the stability of glyphosate-2-^{13}C. Table 3.1 shows the IDMS quantitative comparison of glyphosate after the designated time stability drying conditions. When glyphosate-2-^{13}C is loaded onto the column at a volume of 4.0 mL and allowed to air dry for one week, two weeks, and one month the measured concentration is slightly higher than the calculated concentration of 5.000 ppm. After one week the measured concentration was 6.485±0.35 ppm (n=16). Two weeks and one month of air-drying produced a measured concentration 8.251±0.65 ppm (n=16) and 7.296±0.29 ppm (n=16) respectively.

Air drying the SPE column after the isotopically enriched glyphosate was not stable on the column for one week. Therefore, water and methanol were loaded onto the column after the addition of the spike and allowed to incubate for one week prior to analysis. After one week the measure concentration for water addition was 6.547±0.27 ppm (n=16) and 5.674±0.33 ppm for methanol (n=16). This analysis presented a high bias in the measurement for air drying and maintaining a wet SPE column. Therefore, a reverse i-Spike was performed loading the analyte onto the column prior to the addition of 2.0 mL of methanol and incubation for one week. Upon analysis, the measured concentration was calculated to be 4.357±0.13 ppm (n=16). This is indicative of a transformation of the SPE column after the addition of the first analyte. Tunneling of the SPE column or irreversible binding of the isotopically enriched spike when preloaded onto the column can occur when the SPE column is too wet or too dry.

To increase the stability of the isotope onto the column, 0.22 mL of a concentrated isotope sample was loaded onto the top frit of the SPE packing to not disturb the packing. The samples were incubated for one and two week time points. After one week, the measured concentration was 5.110±0.16 ppm (n=16) and after two weeks 6.330±0.07 ppm (n=16). When loading 0.22 mL of isotope onto the column, the isotope is stable for one week prior to addition of analyte and analysis. To further increase the stability, an individual frit was utilized and put into the SPE cartridge approximately 0.5 cm above the commercialized packing. The columns incubated for one week, two weeks, and one month prior to the addition of the analyte and analysis. After one week the measured

concentration was 5.069±0.58 ppm (n=16). After two weeks and one month the measured concentrations were 5.327±0.38 ppm (n=16) and 7.368±0.10 ppm (n=16) respectively. With the utilization of the individual frit added to the SPE cartridge, the isotope is stable on the cartridge for two weeks. This time was extended from the previously stated time points of zero weeks for air dry and wet columns and one week after the addition of a concentrated isotope sample.

Table 3.1 Time stability study for glyphosate using i-Spike SPE

Drying Conditions	Drying Time	Calculated Conc. (ppm)	Measured Conc. (ppm)	Std. Dev.
Air Dry	1 Week	5.000	6.485±0.35	0.067
Air Dry	2 Week	5.000	8.251±0.65	0.612
Air Dry	1 Month	5.000	7.296±0.29	0.273
2 mL HPLC water	1 Week	5.000	6.547±0.27	0.256
2 mL Methanol	1 Week	5.000	5.674±0.33	0.310
Load analyte 2 mL Methanol	1 Week	5.000	4.357±0.13	0.117
Load 0.22 mL Air Dry	1 Week	5.000	5.110±0.16	0.146
Load 0.22 mL Air Dry	2 Week	5.000	6.330±0.07	0.069
Individual Frit 0.22 mL	1 Week	5.000	5.069±0.58	0.544
Individual Frit 0.22 mL	2 Week	5.000	5.327±0.38	0.357
Individual Frit 0.22 mL	1 Month	5.000	7.368±0.102	0.402

3.4 Conclusions

EPA method 6800 has been extended to the analysis of glyphosate in drinking water samples. The dynamic range analysis of a standardized analyte is required for the proper determination of the ratio range of the analyte. The analysis of a variety of ratios of analyte to isotope using ESI-TOF-MS presented ionization suppression within the sample. When an isotopically labeled analyte is only enriched with a single isotope, suppression occurs with the peak that is of the lower abundance in the mass spectra. This ion suppression was compensated for using an APCI-Q-TOF-MS. With APCI, the

constant electron source from the corona discharge needle decreases ion suppression. The ideal ratio between glyphosate and glyphosate-2-^{13}C is 1:1 but a ratio extreme of 10:1 and 1:10 can be analyzed while still maintaining accuracy and precision.

The optimized ESI-TOF-MS and APCI-Q-TOF-MS methods were utilized for the validation of the two newly developed SPE methods. The SPE-IDMS and i-Spike techniques are comparable to the traditional IDMS quantitation for the analysis of glyphosate in drinking water. Once samples are spiked with the isotopically enriched sample for SPE-IDMS analysis, sample loss and retention, as well as instrument fluctuations and drift do not adversely affect the quantitation. On the other hand, i-Spike has the potential to analyze drinking water samples, de novo, with previously loading the isotopically enriched spike onto the column. With the utilization of an individual frit, the isotope is stable on the SPE column for two weeks prior to analysis.

The limit of quantitation for both SPE-IDMS and i-Spike techniques when the analyte is concentrated onto the SPE column is 0.400 ppm. The detection limit for glyphosate after column concentration is lower then the maximum containment level of 0.7 ppm set by the National Primary Drinking Water Regulations from the USEPA.[11] These techniques have the potential to be employed for rapid and reliable analytical method of glyphosate and other phosphonic acid containing compounds in water samples that does not require time-consuming derivatization or liquid chromatography separation prior to analysis using APCI-Q-TOF-MS. The methods described here are amenable for analyses of a variety of drinking water analytes. Direct sample equilibration SPE-IDMS or by i-Spike, reduced

the biases caused by recovery, calibration and ionization differences without the need of calibration curves and eliminates derivatization. By adapting direct IDMS measurements of glyphosate, future automation and similarly accurate applications for analysis of other molecules, such as pesticides, toxins and toxicants in potable water can be foreseen.

Chapter 4

Quantitation of phosphate based nerve agents in environmental matrices

4.1 Introduction

The identification of chemical weapons is essential for the compliance with the CWC. Although the CWC prohibits the development, production, stockpiling, and use of chemical weapons the threat of use with rogue states and terrorist organizations in still viable.[25] Nerve agents are a class of chemical warfare agent that disrupts neurological regulation by means of inhibiting acetylcholine esterase.[29-31] The threat and dissemination of nerve agents has a two-fold reaction to the public sector. Not only will the release cause mass chaos and death but it will also instill fear into society, which meets the goals of many terrorist organizations.

The detection and quantitation of nerve agents must be coupled with the analysis of their degradation products for verification of use.[29, 37, 42] The hydrolysis of V series and G series nerve agents produce methylphosphonic acid that has a decreased toxicity compared to the original agents.[38] Current techniques for the detection of nerve agents includes analysis by GC-MS[31, 39], LC-MS[30, 37, 40-41], and ion chromatography.[38] There are many drawbacks when using GC-MS, which include sample clean up and a tedious derivatization process prior to analysis. LC-MS analysis is expensive and difficult to operate for a novice individual while ion chromatography has a low specificity and

sensitivity. The use of a calibration curve or internal standard for quantitation is also a drawback with all three techniques described above.

A method for the analysis of nerve agents must be simple, rapid, and reliable. This chapter describes the quantitative analysis of nerve agent surrogates. Two SPE methods have been developed for the analysis of methylphosphonic acid in drinking water samples EPA Method 6800, IDMS, for quantitation.[11]. SPE with an anion exchange resin was used for sample clean up and concentration, utilizing the ionic character of methylphosphonic acid. IDMS is a quantitative analytical technique based on the measurement of isotopic ratios in a sample that has been spiked with a known amount of an isotopically enriched compound. The employment of IDMS in conjunction with SPE eliminates concerns of incomplete recovery and elution, along with potential adverse effects of matrix and sorbent on ionization and mass spectrometer performance. Once equilibration is obtained, any chemical or physical change occurs equally between the species of interest, therefore ionization and most mass spectral errors are reduced or eliminated in significance and accuracy is increased.

This chapter describes two novel methods for the analysis of nerve agent surrogates in drinking water via APCI-Q-TOF-MS that does not involve tedious and time-consuming derivatization. SPE-IDMS and i-Spike were validated for quantitation of surrogates in drinking water. SPE-IDMS involves pre-equilibration of the sample prior to the SPE while i-Spike facilitates the addition of the isotopically enriched analyte onto the SPE column prior to the addition of the sample containing analyte. i-Spike is advantageous

since it enables the isotope to be preloaded onto the column prior to the addition of the analyte, enabling a rapid, simple, and low cost field sample preparation technique. i-Spike has the potential to be useful for applications in forensics, homeland defense, and environmental health assessment.

4.2 Materials and methods

4.2.1 Reagents and supplies

Methylphosphonic acid (99%; 1000 μg/mL in methanol) analytical standard and methylphosphonic acid-^{13}C-methyl-D_3 (^{13}C, 99%; methyl-D_3, 98%; 100 μg/mL in methanol) were purchased from Cerilliant Corporation (Round Rock, TX, USA). HPLC grade water, acetonitrile, and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Bioanalytical grade formic acid was also purchased from Fisher Scientific. The Strata-SAX (500 mg bed mass, 6 mL volume capacity) solid phase extraction columns were purchased from Phenomenex (Torrance, CA, USA). Drinking water was supplied from Pittsburgh municipal water supply (Pittsburgh, PA, USA).

4.2.2 Instrumentation

The Agilent Technologies 6530 Auccurate-Mass quadropule-TOF LC/MS (Santa Clara, CA, USA) was optimized for the analysis of methylphosphonic acid. Analyses were implemented with direct infusion into the APCI source at a flow rate of 0.5 mL/min with a Cole Palmer 74900-00 syringe pump (Vernon Hills, IL, USA). All analyses were completed in negative ionization mode with a mass to charge scan range of 50-200 *m/z*. The optimized APCI-Q-TOF-MS settings are: gas temperature 300 ^{o}C; vaporizer 350 ^{o}C; drying gas 6 L/min; nebulizer 35 psig; capillary voltage 4500 V; corona 18 μA;

fragmentor 115 V; skimmer 65 V; oct 1 rf vpp 110 V. Samples were collected in four replicate samples for a time of 1 minute per replicate for statistical analysis. The peak area and peak abundance for naturally occurring methylphosphonic acid (95.0 *m/z*) and isotopically enriched methylphosphonic acid (99.0 *m/z*) were recorded for each sample for quantitation using IDMS.

4.2.3 Dynamic range analysis

The calculation of the error propagation factor for each analyte determines the ideal ratio between the naturally occurring analyte and the isotopically enriched analyte. Equation 4.1 expresses the determination of the EPF for a given naturally occurring analyte and isotopically enriched isotope.

Equation 4.1. $$EPF = \sqrt{\frac{isotope\ A\ spike\ abundance}{isotope\ B\ spike\ abundance} x \frac{isotope\ A\ natural\ abundance}{isotope\ B\ natural\ abundance}}$$

The optimal spiking ratio for methylphosphonic acid was calculated to be 1.0 when using Equation 3.1. Theoretically, a spike ratio does not have to precisely 1:1. Deviation from the optimal ratio will give an increased error at a determined point. Therefore, a dynamic range analysis was completed for methylphosphonic acid in water.

Samples of methylphosphonic acid were prepared at 1:10, 1:2, 1:1, 2:1, and 10:1 ratio of naturally occurring analyte and isotopically enriched spike. The samples were prepared by mass and analyzed using APCI-Q-TOF-MS and compared for quantitative significance. The peak abundances for both the analyte and spike *m/z* peaks were recorded for IDMS quantitation.

4.2.4 Solid phase extraction method development/validation

Extractions were performed on a Strata-SAX solid phase extraction column with a 500 mg bed mass and a 6.0 mL column volume. SPE-IDMS and i-Spike extraction methods developed in Chapter 3 for glyphosate in drinking water samples were extended to the analysis of methylphosphonic acid. The SPE analyses were completed with a flow rate of 1.0 mL/min maintained by a negative pressure SPE vacuum chamber (Supelco, St. Louis, MO). Comparison analysis between traditional IDMS, SPE-IDMS, and i-Spike were performed with HPLC grade water spiked with both naturally occurring analytes and their isotopically enriched spikes. Comparison analysis was also completed in drinking water samples with SPE-IDMS and i-Spike. Column limit of quantitation and sample concentration were determined in drinking water samples. All analysis was completed using an APCI-Q-TOF-MS.

4.2.4.1 SPE-IDMS

Water samples were spiked by mass at a 1:1 ratio with naturally occurring methylphosphonic acid and isotopically enriched methylphosphonic acid-^{13}C-methyl-D_3 at equivalent concentrations. The Strata-SAX SPE column was conditioned with 4.0 mL of HPLC grade methanol. The column was then equilibrated with 4.0 mL of HPLC grade water. Four grams of equilibrated water sample was then loaded and the column was washed with 4.0 mL of HPLC grade methanol prior to elution with 16.0 mL of 6.0% formic acid in 1:1 acetonitrile:methanol solution. The eluate was analyzed by APCI-Q-TOF-MS. The method was validated by assessing the limit of quantitation, SPE column limit of quantitation, and limit of concentration of analyte onto the SPE column.

To determine the instrument limit of quantitation, water samples were prepared that were spike at a 1:1 ratio of methylphosphonic acid and methylphosphonic acid-^{13}C-methyl-D_3 at decreasing concentrations. The samples were then analyzed via APCI-Q-TOF-MS until the signal to noise ratio was less than 3:1 indicating the limit of detection. The same data was utilized to determine the limit of quantitation. The peak abundances of both analyte (95.0 *m/z*) and isotope (99.0 *m/z*) were utilized to quantify the analyte but IDMS.

To determine the limit of quantitation of the SPE column, drinking water samples were spiked with both the naturally occurring analyte and isotopically enriched spike. Samples were prepared in decreasing concentration and subjected to the SPE procedure. After SPE, the samples were analyzed to determine the lowest quantifiable concentration.

The analytes were also concentrated onto the SPE column to further decrease the limit of quantitation. Samples were spiked at a 1:1 ratio at varying concentrations making sure that enough quantity of sample was prepared to concentrate the sample up to a concentration of 1.00 ppm. After SPE, the samples were analyzed by APCI-Q-TOF-MS.

4.2.4.2 i-Spike

Loading the isotopically enriched spike and naturally occurring analyte individually onto the column by mass completed method development for the i-Spike method. The Strata-SAX column was conditioned with 4.0 mL of HPLC grade methanol and equilibrated with 4.0 mL of HPLC grade water prior to loading the samples. Four grams of isotopically enriched spike was then loaded onto the column followed by loading 4.0 g of naturally occurring analyte at equivalent concentrations. The compounds were

quantitatively transferred onto the column by washing the container and pipette tip used with three 1.0 mL volumes of HPLC water. The SPE column was then washed with 4.0 mL of HPLC grade methanol followed by elution with 16.0 mL of 6.0% formic acid in an acetonitrile:methanol solution. The eluate was analyzed by APCI-Q-TOF-MS. Method validation was completed by determining the limit of quantitation, SPE column limit of quantitation, and limit of concentration of sample onto the SPE column.

To determine the limit of quantitation of the SPE column, drinking water samples were spiked with both the naturally occurring analyte and isotopically enriched analyte. Samples were prepared in decreasing concentration and subjected to the SPE procedure. After SPE, the samples were analyzed to determine the lowest quantifiable concentration.

The analytes were also concentrated onto the SPE column to further decrease the limit of quantitation. Samples were spiked at a 1:1 ratio at varying concentrations making sure that enough quantity of sample was prepared to concentrate the sample up to a concentration of 1.00 ppm. After SPE, the samples were analyzed by APCI-Q-TOF-MS analyzing the peak abundances of the naturally occurring methylphosphonic acid (95.0 *m/z*) and the isotopically enriched methylphosphonic acid-^{13}C-methyl-D_3 (99.0 *m/z*).

4.3 Results and discussion

4.3.1 Instrument method optimization

An optimized method for the analysis methylphosphonic acid in water was developed using APCI-Q-TOF-MS. The method described in Section 4.2.2 had a limit of

quantitation being 0.25 ppm (n=4). The optimized method was utilized for all subsequent analysis of methylphosphonic acid samples.

4.3.2 Dynamic range analysis

The dynamic range analysis was completed for the analysis of methylphosphonic acid by deviating the analyte to spike ratio from the optimal ratio determined from the error propagation factor. The limit of detection for the analysis of methylphosphonic acid was determined to be 0.25 ppm (n=4). Therefore, the concentration analyzed for the dynamic range analysis was 1.00 ppm. The ratios analyzed were 1:10, 1:2, 1:1, 2:1, and 10:1. Figure 4.1 graphically represents the dynamic range analysis for methylphosphonic acid on an APCI-Q-TOF-MS.

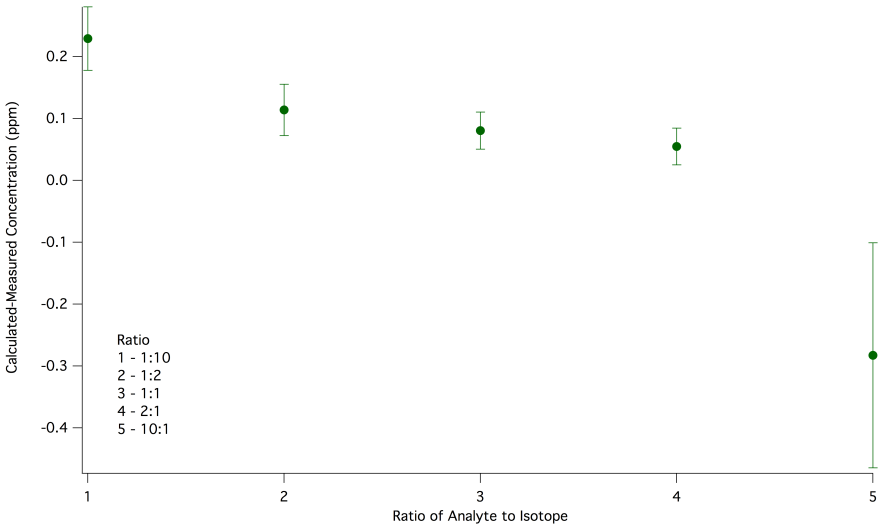


Figure 4.1. Dynamic range analysis of methylphosphonic acid using APCI-Q-TOF-MS

The normalized concentration for the dynamic range analysis was 1.00 ppm. A ratio of 1:1 of the analyte to isotope produced a measured concentration of 0.920±0.03 ppm (n=4). When deviating from the ideal ratio, a 1:10, 1:2, 2:1, and 10:1 ratio produced measured concentrations of 0.771±0.05, 0.886±0.04, 0.945±0.03, and 1.28±0.18 ppm

(n=4) respectively. When the ratio of analyte to isotope is increase to a 1:10 or 10:1 ratio, the error within the measurement is greater than a ±20% error allotted by the USEPA for environmental samples. Therefore, when analyzing an unknown concentration of methylphosphonic acid a ratio no greater than 1:2 or 2:1 can be utilized. From this analysis, it was determined that an unknown methylphosphonic acid sample can precisely and accurately (within 20% error) be quantified using the SPE procedure developed for the analysis of glyphosate. This ratio is also highly dependant on the cleanliness of the mass spectrometer, which will impact background noise of the instrument.

4.3.3 SPE method development/validation

The validation of both SPE-IDMS and i-Spike methods were extended from the analysis of glyphosate to the analysis of methylphosphonic acid. The limit of quantitation was determined to be 0.25 ppm (n=4) in water samples using an APCI-Q-TOF-MS. After the determination of the limit of quantitation the SPE-IDMS and i-Spike methods developed for glyphosate were analyzed using methylphosphonic acid and compared to traditional IDMS of methylphosphonic acid. The comparison of the three sample preparation methods is described in Figure 4.2.

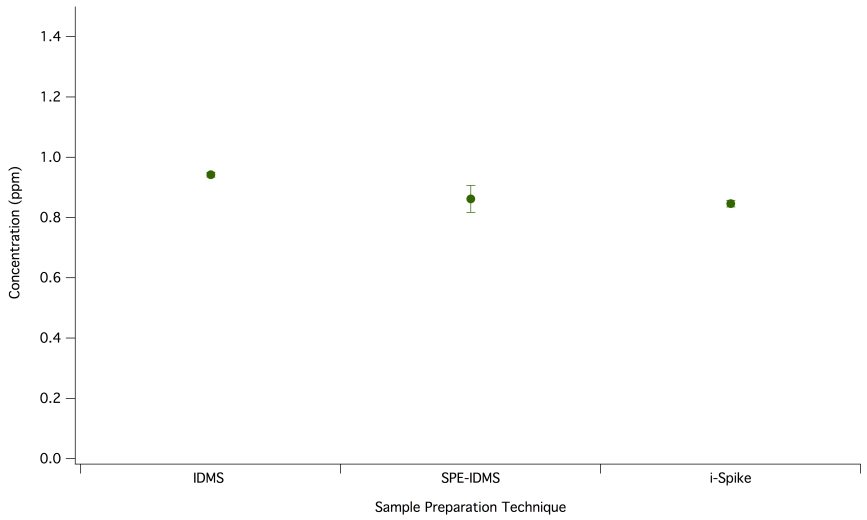


Figure 4.2. Comparison of IDMS, SPE-IDMS, and i-Spike sample preparation methods for methylphosphonic acid using APCI-Q-TOF-MS

The calculated concentration for all sample preparation methods was 1.00 ppm. Traditional IDMS gave a measured concentration of 0.942±0.008 ppm (n=16). SPE-IDMS and i-Spike methodologies had measured concentrations of 0.862±0.045 (n=16) and 0.846±0.011 ppm (n=16) respectively. This data is well within the USEPA standard of a 20% measurement error for a given sample. The measurements are precise but a slightly negatively biased in their accuracy. This may be due to the difference in structure between the glyphosate and methylphosphonic acid. The glyphosate contains a carboxylic acid functional group along with the phosphonic acid function group. Methylphosphonic acid, on the other hand, possesses only a methyl group bound to a phosphonic acid function group. This may have an influence on the retention of the analytes onto the SPE column causing a slight bias in the accuracy for methyphosphonic acid. After validation, SPE column limits of quantitation were determined. The limit of quantitation for methylphosphonic acid on the SPE column for SPE-IDMS and i-Spike

analysis was 0.500 ppm and 0.200 ppm respectively. The SPE column limits of quantitation for SPE-IDMS and i-Spike are represented in Figure 4.5 and Figure 4.6.

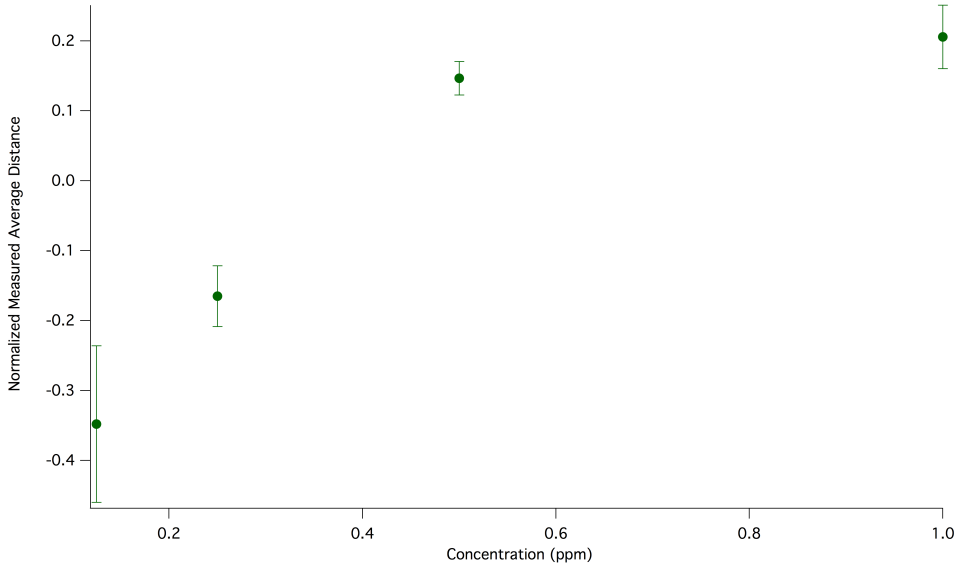


Figure 4.3. SPE column limit of quantitation using SPE-IDMS technique

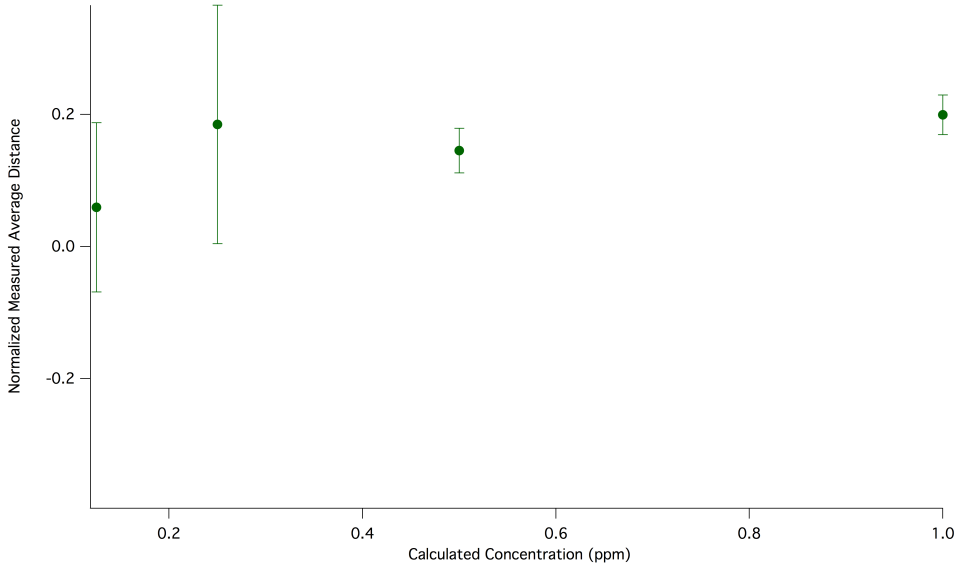


Figure 4.4. SPE column limit of quantitation using i-Spike technique

To further decrease the limit of quantitation, the methylphosphonic acid was concentrated onto the SPE column using the SPE-IDMS method as described in Figure 4.5. When

loading a lower concentration of the analyte onto the column a larger mass was loaded to load the analyte up to a concentration of 1.00 ppm. The lowest concentration that could be loaded onto the column up to a concentration of 1.00 ppm was 0.031 ppm. The amount of analyte that is capable of being loaded onto the column was not dependant on the bed mass in these samples but rather the over abundance of water loaded onto the column that caused for the column packing to be washed from the column.

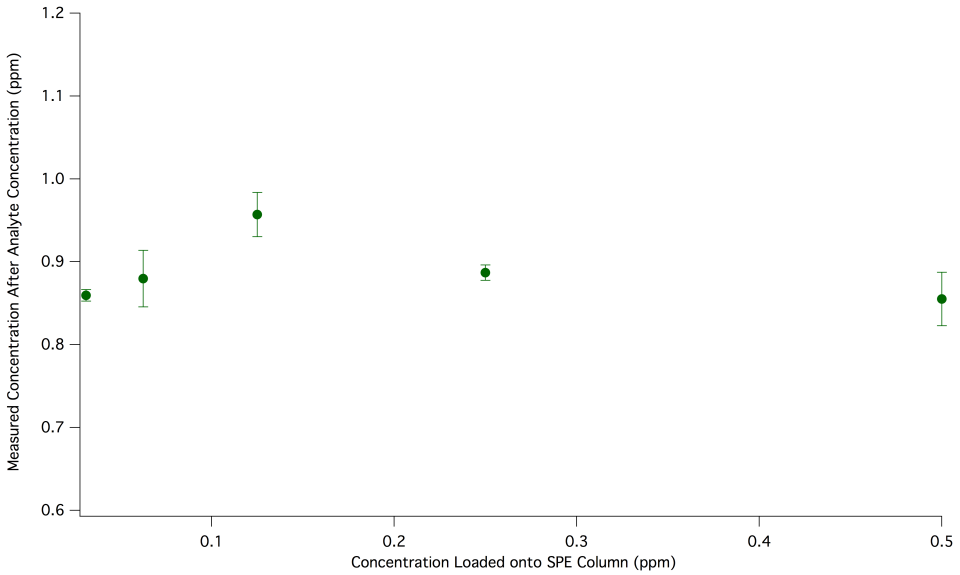


Figure 4.5. Methylphosphonic acid concentration onto SPE column using SPE-IDMS

After method validation samples were prepared that contained both glyphosate and methylphosphonic acid and analyzed by the solid phase extraction methods. Figure 4.6 shows the simultaneous anlaysis of glyphosate and methylphosphonic acid in water samples using SPE-IDMS with analysis on an APCI-Q-TOF-MS. Glyphosate was prepared with a calculated concentration of 6.00 ppm and yielded a measured concentration of 5.770±0.113 ppm (n=4). The methylphosphonic acid in the sample was prepared with a calculated concentration of 1.00 ppm and yielded a measured concentration 0.880±0.059 ppm (n=4). These two newly developed methods described

have the ability to analyze a mixture of phosphonic acid containing compounds in drinking water samples with the accuracy and precision required by the USEPA.

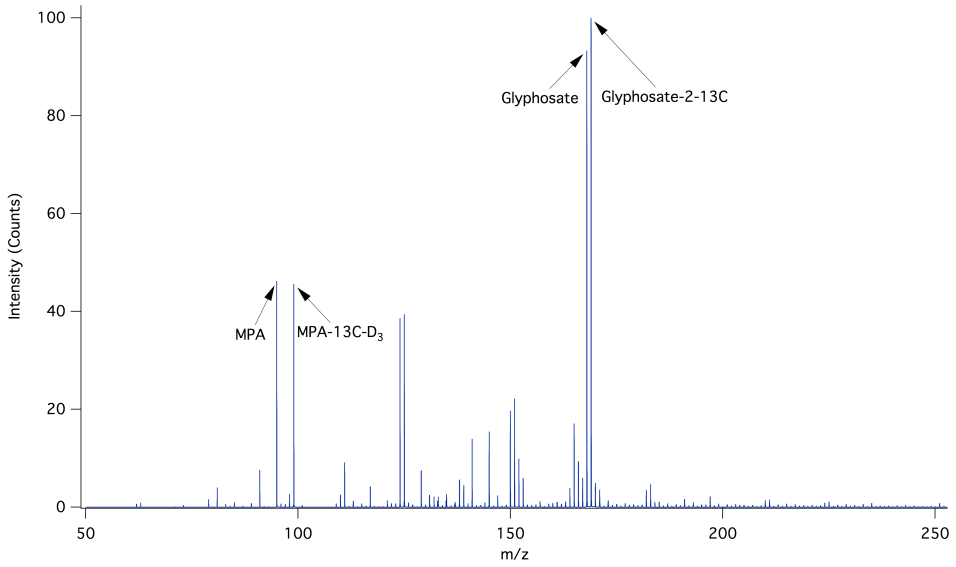


Figure 4.6. Simultaneous analysis of glyphosate and methylphosphonic acid in water samples using SPE-IDMS

4.4 Conclusions

The SPE-IDMS and i-Spike methods developed for the analysis of glyphosate in drinking water has successfully been extended to the analysis of methylphosphonic acid in water samples. Analysis was completed on an APCI-Q-TOF-MS with a limit of quantitation of 0.25 ppm. The SPE-IDMS and i-Spike methods were assessed against traditional IDMS analysis to determine validity of the methods. Traditional IDMS had a measured concentration of 0.942±0.008 ppm (n=16) compared to a normalized calculated concentration of 1.00 ppm. SPE-IDMS and i-Spike methodologies had measured concentrations of 0.862±0.045 (n=16) and 0.846±0.011 ppm (n=16) respectively. This data is well within the USEPA standard of a 20% measurement error for a given sample. The slight bias of the measurements may be explained by the difference in retention

between the glyphosate and the methylphosphonic acid in the SPE column with the established methods.

The limit of quantitation for the SPE columns for both SPE-IDMS and i-Spike methods were investigated to further validate the sample preparation methods. SPE-IDMS had a limit of quantitation of 0.500 ppm and 0.200 ppm respectively. To further decrease the limit of quantitation for methylphosphonic acid, the analyte was concentrated onto the SPE column. When concentrating methylphosphonic acid, the limit of quantitation was extended to 0.031 ppm.

To determine the applicability of the SPE-IDMS and i-Spike methods for the analysis of phosphonic acid based nerve agents (V-series and G-series) and environmental samples, glyphosate and methylphosphonic acid were quantitatively assessed simultaneously. Both glyphosate and methylphosphonic acid quantitation were precise with measured concentrations of 5.770±0.113 ppm (n=4) and 0.880±0.059 ppm (n=4) respectively. The calculated concentration for glyphosate was 6.00 ppm and 1.00 ppm for methylphosphonic acid. This preliminary data suggests that the two newly developed SPE techniques have the ability to assess samples containing multiple phosphonic acid containing compounds.

Chapter 5

Alternative method for the quantitation of illicit drugs, metabolites, and contaminants in urine correcting for metabolism

5.1 Introduction

Opiates and/or opioids are described as any natural or synthetic drug with morphine like properties.[47, 58] The opiate classification includes opium, morphine, diacetylmorphine, methadone, and codeine. Heroin, a semi synthetic morphine derivative, was developed in 1874 by A.C. Wright to increase the potency of morphine.[48-50] The increased potency is due to its higher lipophobicity compared to morphine enabling heroin to cross the blood brain barrier at a faster rate.[50-51] Heroin is rapidly metabolized by serum and liver esterase activity into 6-actylmorphine and subsequently morphine. The clearance of morphine occurs via glucuronidation in the liver, predominantly to morphine-3-glucuronide and morphine-6-glucuronide.[52-55] The elimination half-life of heroin is between two and eight minutes.[48-50, 55] Due to the rapid metabolism of heroin, unequivocal proof of heroin use requires the identification of 6-acetylmorphine in blood or urine.[56] The half-life of 6-acetylmorphine is between six and twenty-five minutes in blood and eight hours in urine.[49-50, 52, 57] 6-Acetylmorphine is then metabolized to morphine, which possesses an elimination half-life of 40 minutes.[55, 57]

Detection of opiates not only includes the detection of the drug itself but also its metabolites for unequivocal identification of the abused drug. Another aspect of illicit

drug use that complicates drug identification is the addition of contaminants or combination drug use. Contaminants are components that are added to the illicit drug to either increase or decrease the effect of the main drug component.[58] Contaminants are also used as "bulking-agents" to increase the quantity sold but decreasing the overall amount of illicit drug present. In 2006, the Philadelphia Medical Examiner's Office reported the detection of xylazine and fentanyl in drug-related cases. Also in 2006, there were several cities in the United States that reported increased mortality rates due to fentanyl. These cases were determined to be cocaine and heroin samples that were "contaminated" with fentanyl.[59] Fentanyl is used medically in the treatment of severe chronic pain and is estimated to be 30-50 times more potent than heroin.[59] Other than fentanyl common contaminants include heroin, cocaine, codeine, procaine, and quinine.[58]

Another aspect that may complicate the analytical detection of illicit drug use is if the program subject is in drug treatment therapy. Methadone has been one of the most widely used methods to treat the withdrawal symptoms associated with opiates since 1950.[60] Often times even if a patient is associated with a methadone treatment program, they continue to use illicit drugs. Fatalities due to methadone use greatly increased in the early 2000's. The majority of the methadone related deaths included the use of another opiate or central nervous system depressant.[60] Although methadone treatment programs are the most common form of opiate dependence treatment in the United States, a few European countries have pharmaceutical heroin treatment programs for heroin addiction. This treatment program is for individuals that fail to respond to the more traditional methadone treatment.[6] This imposes yet another layer into the complicated process of

illicit drug detection. Pharmaceutical heroin possesses the same metabolites in blood and urine as illicit heroin. Therefore, the identification of impurities in the illicit production of heroin must be identified to provide information on illicit heroin use while in a pharmaceutical heroin treatment program. One of the most common impurities in heroin production is acetylcodeine, which has been reported to be present in 86% of urine samples containing illicit heroin.[6, 61]

Current analysis protocols describe initial screening of illicit drugs by immunological methods.[62-63] Immunological methods are limited in their screening for a wide range of drugs, high cost of reagents, and high incidence of false negatives.[62, 64-65] These limitations are a significant downfall in the current detection of illicit drugs. Currently, if a positive immunological test is not present the sample does not progress into analytical analysis and identification. Although these techniques are sensitive, if the tests does not screen for the drug that is present, a negative result ensues and the sample does not go onto analytical identification.

Positive immunological samples undergo quantitative analytical analysis. Blood and urine samples are typically quantitatively assessed by internal standard or calibration curve techniques.[58-59, 66-67] Most analysis techniques require a sample clean up step to remove matrix interferences. These techniques include SPE, LLE, and protein precipitation.[58-59, 65-66, 68-70] GC-MS analysis of illicit drugs was the gold standard until the advent of LC-MS/MS.[71-73] The analysis of illicit drugs by GC-MS typically involves a tedious and time consuming process of sample clean up followed by analyte

derivatization to increase volatility for analysis.[51, 62, 64, 66-68, 74-75] Unlike GC-MS methods, LC-MS/MS methods do not require derivatization of the illicit drugs prior to analysis and are typically analyzed in selected ion monitoring mode to increase sensitivity.[47, 64, 71] LC-MS/MS still requires sample preparation and matrix effects of samples must still be accounted for.[64, 68, 71] Other advantages of LC-MS/MS over the traditional GC-MS analysis are that the sample can be analyzed in a scan mode or a multiple reaction monitoring mode to select for ions and identify parent ions.[69]

Although LC-MS/MS is a selective technique, quantitative identification may become difficult with matrix interferences and a large quantity of analytes to identify. Another downfall of LC-MS/MS analysis is that most internal standards used for quantitation of drugs are deuterium labeled analogues of the drugs themselves. Although deuterium labeled analogues are currently used for quantitation with a mass spectrometer, the physical and chemical similarities often times causes co-elution or poor resolution in liquid chromatography. A simultaneous method for the quantitative analysis of heroin, 6-acetylmorphine, morphine, cocaine, codeine, fentanyl, and methadone is described using two newly developed SPE techniques followed by analysis using ESI-TOF-MS. The resolution on the TOF-MS is significantly greater than the unit mass resolution of the LC-MS/MS decreasing the opportunity of interferences from the matrix.[76] SPE-IDMS involves pre-equilibration of the sample prior to the SPE while i-Spike facilitates the addition of the isotopically enriched analyte onto the SPE column prior to the addition of the sample containing analyte. i-Spike is advantageous since it enables the isotope to be preloaded onto the column prior to the addition of the analyte, enabling a rapid, simple,

and low cost field sample preparation technique. Quantitative assessment was completed using IDMS.[42] IDMS is a quantitative analytical technique based on the measurement of isotopic ratios in a sample that has been spiked with a known amount of an isotopically enriched compound. The relationship between the naturally occurring analyte and the isotopically enriched spike are mathematically expressed using the isotope dilution equation. The employment of IDMS in conjunction with SPE eliminates concerns of incomplete elution, along with matrix and sorbent effects. Once equilibration is obtained, any chemical or physical change occurs equally between the two species. IDMS in conjunction with ESI-TOF-MS enables greater resolution and increased accuracy compared to the current GC-MS and LC-MS/MS techniques. These newly developed analytical methods do not require derivatization or separation with liquid chromatography prior to analysis with ESI-TOF-MS due to the resolving power of the TOF-MS and the ionization potential of the ESI ionization source.

5.2 Materials and methods

5.2.1 Reagents and supplies

Heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl analytical standards were purchased from Cerilliant (Round Rock, Texas, USA). The respective deuterium labeled counterparts, heroin-D_9, 6-acetylmorphine-D_3, morphine-D_3, cocaine-D_3, codeine-D_3, methadone-D_3, and fentanyl-D_5 were also purchased from Cerilliant. Synthetic urine, HPLC grade methanol, HPLC grade water, Hyclone phosphate buffered saline, HPLC grade 2-propanol, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetate buffer was prepared using sodium acetate and acetic acid purchased from Fisher Scientific. UCT (Bristol, PA,

USA) Clean Screen™ CSDAU303 (300 mg bed mass, 3 mL volume) SPE cartridges were used for the extraction of the drugs from synthetic urine.

5.2.2 Instrumentation

A Bruker Daltonics microTOF (Billerica, MA, USA) mass spectrometer with an orthogonal ESI source was optimized for the analysis of heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, fentanyl, and their respective deuterium enriched analogues. Analyses were implemented with direct infusion into the ESI source at a flow rate of 240 μL/hour with a Cole Palmer 74900-00 syringe pump (Vernon Hill, Il, USA). All analyses were completed in positive ionization mode with a mass to charge range of 240-400 *m/z*. The optimized instrumental parameters for all seven drugs is as follows: endplate offset -500 V; capillary voltage -4500 V; nebulizer 0.4 Bar; dry gas 4.0 L/min; dry temperature 200 °C; capillary exit 135 V; skimmer 1 40.0 V; hexapole 1 23.0 V; hexapole RF 250.0 Vpp; skimmer 2 24.0 V. The peak areas and abundances were recorded for the naturally occurring heroin (370 *m/z*), 6-acetylmorphine (328 *m/z*), morphine (286 *m/z*), cocaine (304 *m/z*), codeine (300 *m/z*), methadone (310 *m/z*), and fentanyl (337*m/z*). The deuterium labeled analogues were also analyzed for their peak areas and intensities; heroin-D_9 (379 *m/z*), 6-acetylmorphing-D_3 (331 *m/z*), morphine-D_3 (289 *m/z*), cocaine-D_3 (307 *m/z*), codeine-D_3 (303 *m/z*), methadone-D_3 (313 *m/z*), and fentanyl-D_5 (342 *m/z*).

5.2.3 Solid phase extraction method development/validation

Extractions were performed on an UCT Cleen Screen CSDAU303 SPE column with a 300 mg bed mass and a 3.0 mL column volume. SPE-IDMS and i-Spike SPE methods were developed for the analysis in urine. The SPE analyses were completed with a flow

rate 1.0 mL/min maintained by a negative pressure SPE vacuum chamber (Supelco, St. Louis, MO). Comparison analysis between SPE-IDMS, and i-Spike were performed with synthetic urine spiked with both naturally occurring analytes and their isotopically enriched isotopes. Column limits of quantification and sample concentration limits were also determined in urine samples.

The relative response factor of each analyte and isotopically enriched analogue was determined for optimal accuracy. Each drug was spike at a 1:1 ratio with its isotopic analogue and analyzed using ESI-TOF-MS. The instrumental response of the analyte and the isotope were recorded as well as the concentrations. These values were used to determine the response factor of each illicit drug according to Equation 5.1.

Equation 5.1 $$RRF = \frac{(A_c)(C_{is})}{(A_{is})(C_c)}$$

Where A_c is the response of the analyte and A_{is} is the response of the isotope. The concentration of the analyte and isotope are represented by variables C_c and C_{is}, respectively. The manipulation of Equation 5.1 demonstrates that the internal standard is directly proportional to the isotopes intensity and concentration as shown in Equation 5.2.

Equation 5.2 $$\left(\frac{A_c}{C_c}\right) = RRF\left(\frac{A_{is}}{Ci_s}\right)$$

The relative response factor for each analyte was determined and subsequently multiplied to the isotope intensities for in a future analysis.

5.2.3.1 SPE-IDMS

A SPE method was developed for the drugs and their deuterium enriched spikes in urine samples. The optimized procedure first conditions the CSDAU303 column with 2.0 mL of methanol, 2.0 mL water, and 2.0 mL phosphate buffer (pH 6.0). The sample containing 4.0 mL 100 ng/mL drug standard spiked at a 1:1 ratio with the deuterium analogue and 3.0 mL of phosphate buffer was then loaded onto the SPE column at a volume to 4.0 mL. The column was washed with 4.0 mL water, 3.0 mL acetate buffer (pH 4.5), 3.0 mL of methanol, and 1.0 mL eluate. After the column dried for two minutes the sample was elute from the column with 11.0 mL ethyl acetate:2-propanol:ammonium hydroxide (84:12:1). To determine the volume of eluate required for each individual drug, a SPE was completed collecting twelve 1.0 mL volumes of eluate. Each drug underwent the same SPE procedure and each fraction was analyzed with the optimized ESI-TOF-MS method.

The optimized eluate volume was then utilized to simultaneously analyze all seven drugs after equilibration with the isotopically enriched analogues using SPE-IDMS. Urine samples were spiked at a 1:1 ratio with the naturally occurring drugs and their deuterium enriched counterparts at a concentration of 50 ng/ml. The spiked urine sample was then diluted at a 3:1 ratio with phosphate buffer. The sample then underwent the optimized SPE-IDMS procedure. The limit of quantitation of the SPE-IDMS method was assessed by analyzing samples at decreasing concentrations. Concentrations of 100, 50, 25, 12.5, 6.25, and 3.25 ng/mL were analyzed by SPE-IDMS to determine the limit of quantitation.

To further decrease the limit of quantitation for the analysis of the drugs in urine, samples were concentrated onto the SPE column. When concentrating the analytes onto the column, a decreased concentration of analyte required an increased volume of analyte to concentrate the sample to a concentration of 25 ng/mL. Sample concentrations of 25, 12.5, 6.25, 3.13, and 1.56 ng/mL were concentrated onto the SPE column to a final concentration of 25 ng/mL and compared.

Upon the determination of the limit of quantitation, a dynamic range study was performed on all analytes. The optimal spiking ratio of all seven drugs were calculated to be 1.0. With the error propagation factor calculation, theoretically a spike ratio does not have to be precisely a 1:1 ratio. Deviation from the ratio will give an increased error at a determined point. Therefore, a dynamic range analysis was completed for all analytes at varying ratios.

Samples of all analytes were prepared at 1:10, 1:2, 1:1, 2:1, and 10:1 ratios of naturally occurring analyte and isotopically enriched spike. The samples were prepared by mass and then analyzed on the ESI-TOF-MS by the previously optimized method. The peak abundances of both the analytes and spikes *m/z* peaks were recorded for IDMS quantitation.

5.2.3.2 i-Spike

The SPE-IDMS technique was extended to the i-Spike method development. The i-Spike technique involves loading the naturally occurring analyte and isotopically enriched spike onto the SPE column independently. Upon elution, the analyte and spike are equilibrated

and analyzed on the ESI-TOF-MS. The Cleen Screen CSDAU303 column was conditioned with 2.0 mL of methanol, 2.0 mL water, and 2.0 mL phosphate buffer (pH 6.0). A 4.0 mL isotopically enriched urine sample was buffered with 3.0 mL of phosphate buffer and then loaded onto the column at a volume of 4.0 mL. The naturally occurring analytes were prepared in the same manner as the spike, loading 4.0 mL of buffered urine sample onto the SPE column. Both the spike and the analyte were quantitatively transferred onto the column. After the sample was loaded, two 1.0 mL volumes of phosphate buffer were used to rinse the sample container. The SPE column was then washed with 4.0 mL water, 3.0 mL acetate buffer (pH 4.5), 3.0 mL of methanol, and 1.0 mL of eluate. After the column dried for two minutes the sample was elute from the column with 11.0 mL ethyl acetate:2-propanol:ammonium hydroxide (84:12:1).

To determine the limit of quantitation of the i-Spike method, synthetic urine samples were prepared with decreasing concentrations of naturally occurring drugs and isotopically enriched spikes. Sample concentrations of 200, 100, 50, 25, 12.5, 6.25, and 3.13 ng/mL were subject to the i-Spike SPE procedure and analyzed using ESI-TOF-MS.

5.2.1 Stability assessment of i-Spike pre-loaded column

The length of stability of pre-loading each isotope onto the SPE column was determined by allowing the isotopes to be loaded onto the column and incubate for a designated time period prior to analyte loading the analyte and analysis. The deuterium labeled isotopes for each drug were prepared at a concentration of 8.0 μg/mL in phosphate buffered saline. The SPE column was prepared by placing a frit approximately 0.5 cm above the column packing. The isotope solution was then loaded onto the column at a mass of 20.0

mg and allowed to air dry for one week and two week time increments. For each designated time point, four SPE columns were prepared for the time stability analysis.

Prior to analysis, illicit drug samples in urine were prepared at a concentration of 80.0 ng/mL. Four grams of sample was then taken and diluted with 3.00 g of phosphate buffered saline to stabilize the pH of the analytes prior to column loading. The optimized SPE method was then used to load and analyze the samples. To condition the column, 2.0 mL of methanol followed by 2.0 mL of water and 2.0 mL of phosphate buffered saline were loaded onto the column. The phosphate buffered saline diluted analyte sample was then loaded onto the column at a mass of 4.00 g. A quantitative transfer was completed to ensure complete transfer of the analytes to the column. The column was then washed with 4.0 mL water, 3.0 mL acetate buffer (pH 4.5), 3.0 mL methanol, and 1.0 mL eluate. The columns were allowed to dry for two minutes prior to elution with 11.0 mL (84:12:1) ethyl acetate:2-propanol:ammonium hydroxide. The eluate was then directly analyzed without derivatization by ESI-TOF-MS.

5.3 Results and discussion

5.3.1 Illicit drug SPE method development/validation

A method was developed on an ESI-TOF-MS for the analysis of illicit drugs in urine samples. All analyses were completed in positive ionization mode with a mass to charge range of 240-400 *m/z*. The optimized instrumental parameters for all seven drugs is as follows: endplate offset -500 V; capillary voltage -4500 V; nebulizer 0.4 Bar; dry gas 4.0 L/min; dry temperature 200 °C; capillary exit 135 V; skimmer 1 40.0 V; hexapole 1 23.0 V; hexapole RF 250.0 Vpp; skimmer 2 24.0 V. Figure 5.1 shows a ESI-TOF-MS mass

spectra of all seven drugs spiked at a 1:1 ratio with their isotopically enriched analogue. All analytes and spikes are resolved in the mass spectra enabling simultaneous identification and quantitation of each drug.

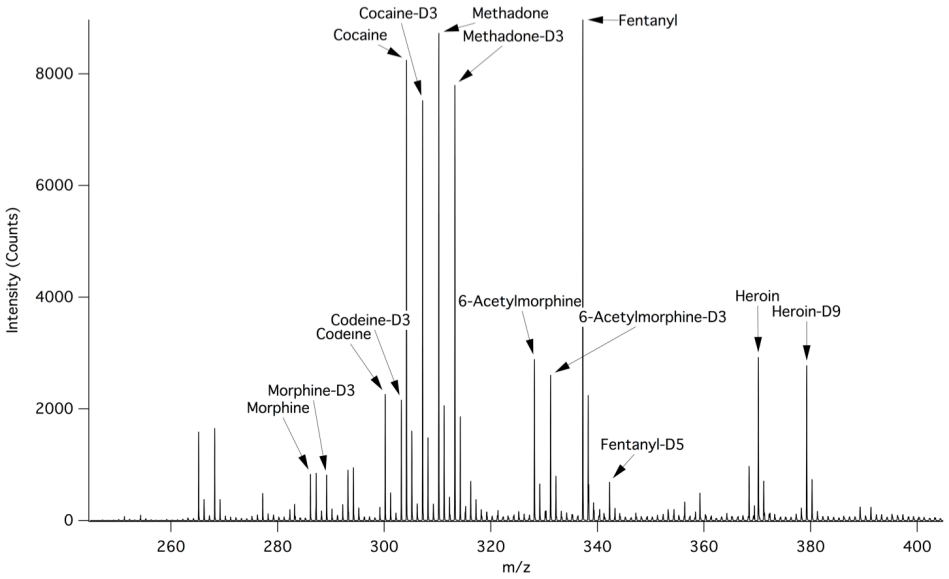


Figure 5.1 Mass spectra of heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl spiked at a 1:1 ratio with their isotopically enriched analogues

5.3.1.1 Relative response factor

The relative response factor was determined for heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl. IDMS quantitation was completed on each analyte prior to the determination of the relative response factor as depicted in Table 5.1 where n=28.

Table 5.1. IDMS analysis of illicit drugs prior to relative response factor determination

Drug	Calculated Concentration (ng/mL)	Measured Concentration (ng/mL)	Standard Deviation	%RSD
Heroin	40.000	40.153±4.084	7.961	19.82
6-Acetylmorphine	40.000	42.569±3.001	5.849	13.74
Morphine	40.000	35.699±0.674	0.674	1.89
Cocaine	40.000	34.157±0.933	1.819	5.33
Methadone	40.000	55.822±8.861	17.273	30.94
Fentanyl	40.000	301.370±58.278	113.602	37.70
Codeine	40.000	41.202±1.172	2.285	5.55

The quantitation of fentanyl produces the highest percent relative standard deviation when compared to the calculated concentration. The percent relative standard deviation for fentanyl was 37.70% while all other analytes other than methadone were within the generally accepted 20% relative standard deviation. When the analyte and isotope are analyzed individually the signal intensity of the isotope is significantly lower than the analyte signal intensity. This is depicted in Figure 5.2 showing an overlay mass spectra of fentanyl, fentanyl-D_5, and a 1:1 ratio of fentanyl:fentanyl-D_5. The difference in ionization between the analyte and isotope is not due to ion suppression but rather the location of the deuterium on the isotopic spike. The deuterium is located on the aromatic ring of the fentanyl causing a change in structural confirmation and subsequently decreasing the ionization potential.

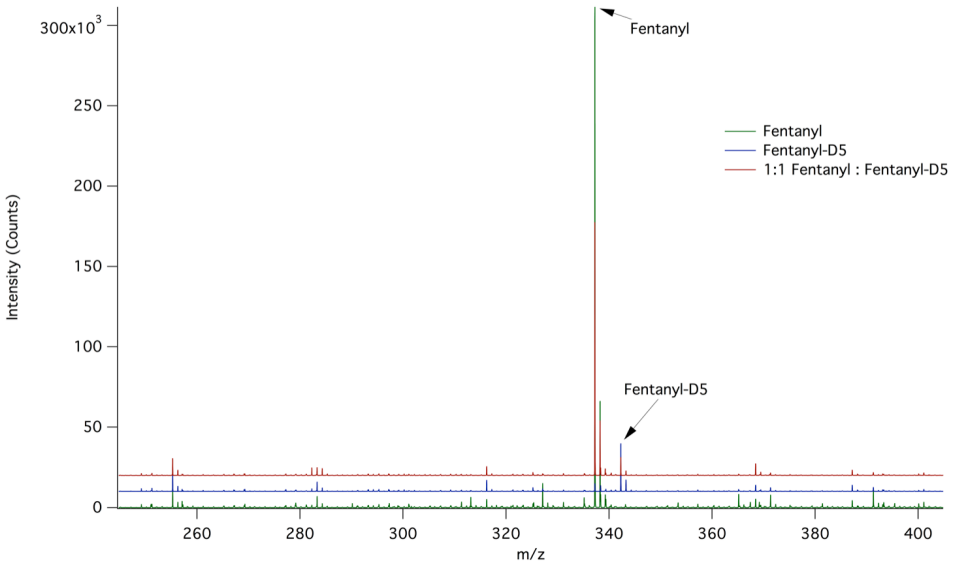


Figure 5.2. Mass spectral analysis of fentanyl and fentanyl-D_5

To correct for the ionization differences between the analytes and isotopic spikes, the relative response factor was determined for each analyte. The relative response factors are depicted in Table 5.2. The relative response factors were applied to the isotope signals and IDMS quantitation was completed.

Table 5.2. Relative response factor for seven illict drugs

Drug	Relative Response Factor
Heroin	0.968
6-Acetylmorphine	1.099
Morphine	0.962
Cocaine	0.943
Methadone	1.220
Fentanyl	9.510
Codeine	1.062

The corrected IDMS analysis is shown in Table 5.3 where n=28. There was a significant change in the measured concentration of fentanyl after the utilization of the relative response factor. The relative standard deviation decreased from 37.70% to 7.16% with

the utilization of the relative response factor. The use of the relative response factor corrects for differences in the ionization potentials between the analytes and their isotopically labeled analogues.

Table 5.3 IDMS of seven illicit drugs will response factor analysis

Drug	Calculated Concentration (ng/mL)	Measured Concentration (ng/mL)	Standard Deviation	%RSD
Heroin	40.000	41.476±4.219	8.224	19.83
6-Acetylmorphine	40.000	38.744±2.731	5.324	13.74
Morphine	40.000	37.576±0.974	1.899	5.05
Cocaine	40.000	36.234±0.990	1.930	5.33
Methadone	40.000	45.323±7.296	14.222	31.38
Fentanyl	40.000	35.753±1.826	2.559	7.16
Codeine	40.000	38.807±1.104	2.152	5.55

5.3.1.2 SPE-IDMS and i-Spike

Two SPE methods were developed for the quantitative analysis of heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl using IDMS that does not require derivatization prior to analysis. A total of twelve 1.0 mL fractions were collected from the elution step for the SPE analysis of the individual drugs to determine the total elution volume. Figure 5.3 illustrates the fraction collection of all seven drugs at a concentration of 50 ng/mL with the same elution solution. Figure 5.4 illustrates the fraction collection of all seven drugs at a concentration of 100 ng/mL with the same elution solution.

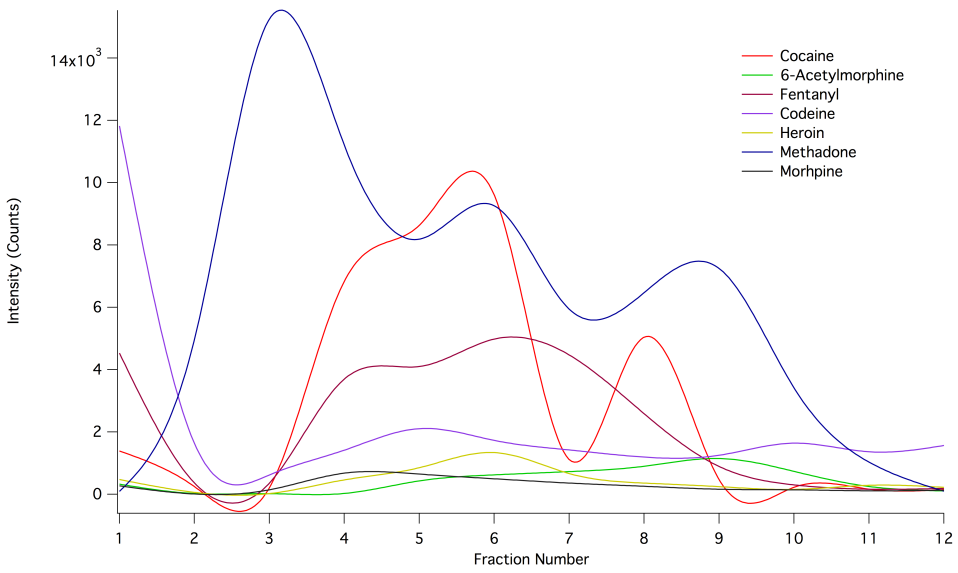


Figure 5.3 Elution volume analysis for all seven drugs with a concentration of 50 ng/mL

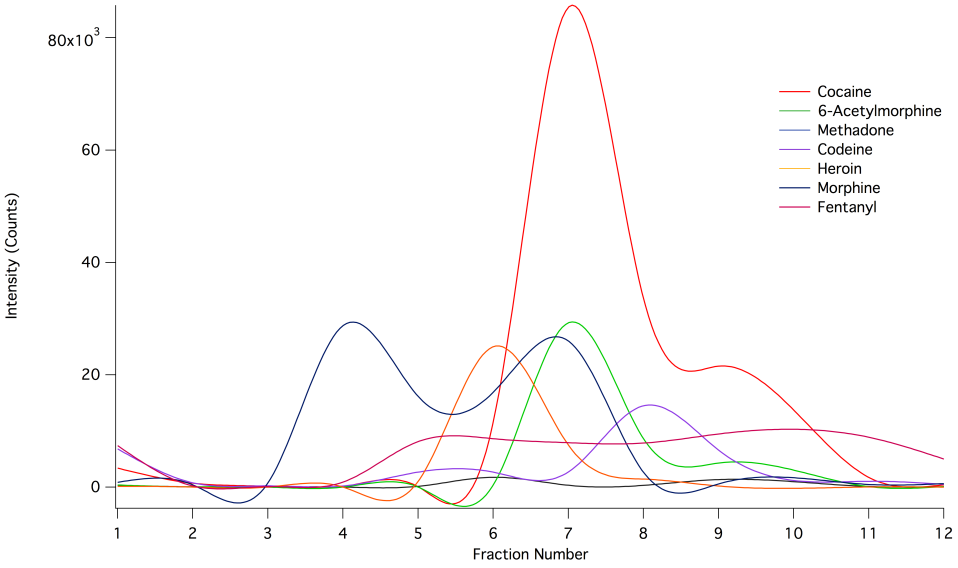


Figure 5.4 Elution volume evaluation for all seven drugs with a concentration of 100 ng/mL

The first 1.0 mL fraction does not contain any of the drugs and has a high background noise. This is due to the continued flushing of the wash steps of the SPE protocol. It is also noted that all seven drugs were fully eluted from the SPE column after a total of 12.0 mL. Therefore, the optimized elution volume is 11.0 mL after 1.0 mL of eluate is loaded

onto the column and discarded. It is imperative with i-Spike to fully elute all analytes and isotopically enriched spikes from the column for proper quantitation. This elution volume remained constant for the remaining experiments.

After both SPE-IDMS and i-Spike methods were optimized, synthetic urine samples spiked at a 1:1 ratio of both naturally occurring drug and isotopically enriched drug was analyzed. Both SPE methods were compared for quantitative validity. Table 5.4 describes the IDMS quantitation of all seven drugs using the SPE-IDMS (n=28) technique. The percent relative standard deviations for all analytes except for methadone were well within the accepted standard deviation of 20%.

Table 5.4. SPE-IDMS analysis of seven drugs in synthetic urine samples

Drug	Calculated Concentration (ng/mL)	Measured Concentration (ng/mL)	Standard Deviation	%RSD
Heroin	40.000	41.476±4.219	8.224	19.83
6-Acetylmorphine	40.000	38.744±2.731	5.324	13.74
Morphine	40.000	37.576±0.974	1.899	5.05
Cocaine	40.000	36.234±0.990	1.930	5.33
Methadone	40.000	45.323±7.296	14.222	31.38
Fentanyl	40.000	35.753±1.826	2.559	7.16
Codeine	40.000	38.807±1.104	2.152	5.55

Table 5.5 describes IDMS quantitation of all seven drugs using the i-Spike (n=16) technique. The normalized calculated concentration for each sample was 40.00 ng/mL. All seven drugs were within a 10% relative standard deviation as depicted in Figure 5.5. Figure 5.5 compares the normalized calculated concentration of each drug with the normalized measured concentration for both SPE-IDMS and i-Spike. The two methods give comparable results with each other and are within a ±10% error.

Table 5.5. i-Spike quantitation of seven drugs in synthetic urine samples

Drug	Calculated Concentration (ng/mL)	Measured Concentration (ng/mL)	Standard Deviation	%RSD
Heroin	40.000	42.166±1.878	3.523	8.36
6-Acetylmorphine	40.000	39.692±2.312	4.337	10.93
Morphine	40.000	39.580±1.297	2.434	6.15
Cocaine	40.000	44.061±1.004	1.883	4.27
Methadone	40.000	32.751±0.820	1.538	4.70
Fentanyl	40.000	44.543±1.976	3.707	8.32
Codeine	40.000	37.989±0.813	1.525	4.01

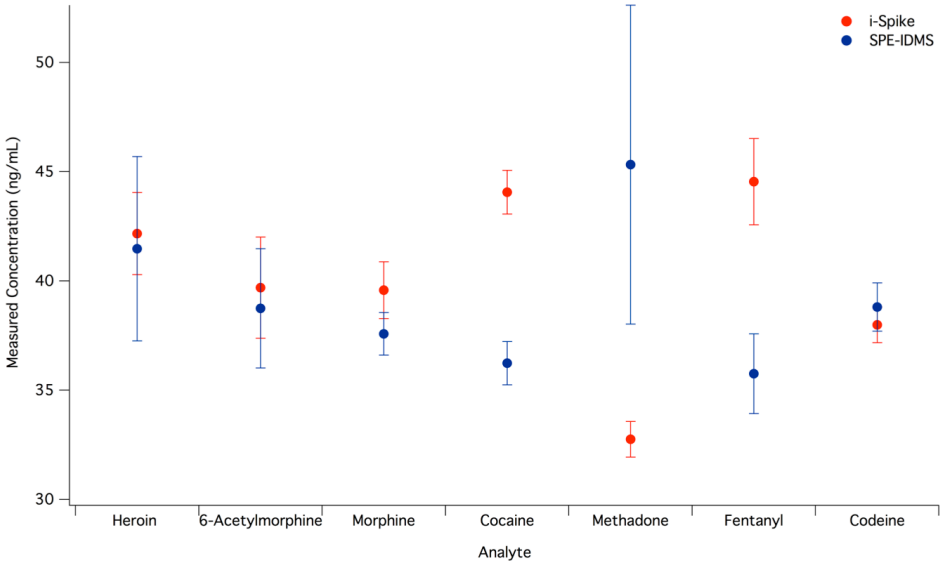


Figure 5.5. Comparison of SPE-IDMS and i-Spike for seven illicit drugs

Upon method optimization, a dynamic range analysis was completed with the SPE-IDMS method. The ratio of naturally occurring drug and isotopically enriched drug were varied from the optimal spiking ratio. The ratios analyzed were 10:1, 2:1, 1:1, 1:2, and 1:10. The analysis was completed for all seven drugs with results described in Figure 5.6. Figure 5.6 shows the deviation of the measured concentrations from the calculated

concentration. A y-axis value of zero would indicate no deviation from the calculated concentration. The ratio range from 10:1 and 1:10 for each drug other than methadone are within a 20% relative standard deviation. The farther the ratio deviates from the optimal spiking ratio the greater the error is within the measurement. Therefore, analysis of the drugs should be completed with no greater than a 10:1 or 1:10 ratio of analyte to spike.

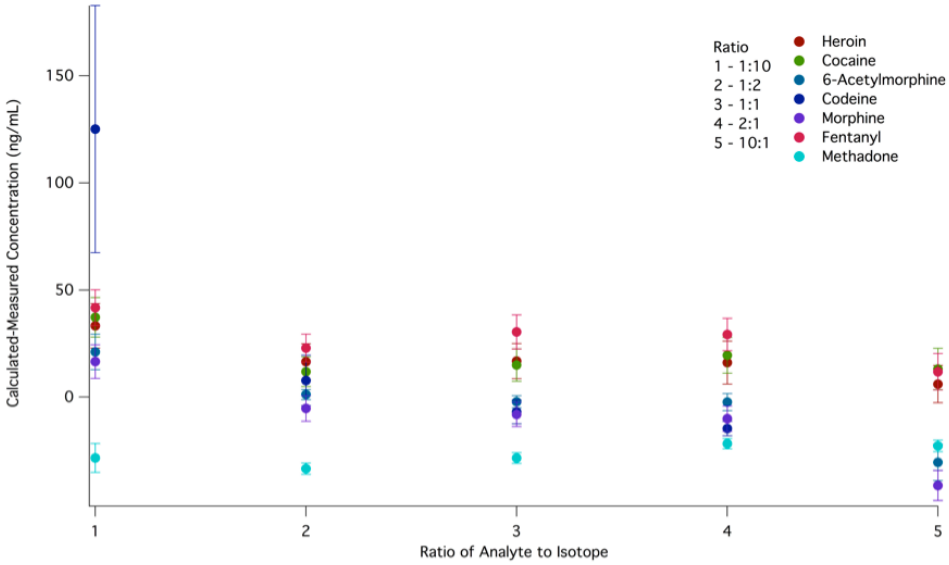


Figure 5.6. Dynamic range analysis of illicit drugs using SPE-IDMS

The limits of quantitation were determined for both SPE-IDMS and i-Spike. Equilibrated urine samples containing all seven drugs and their isotopically enriched analogues were analyzed at decreasing concentrations for SPE-IDMS detection. Concentrations of 1500, 750, 375, 187.5, 93.6, 46.9, 23.4, 11.7, 5.9, and 2.9 ng/mL were analyzed to determine the limit of quantitation (n=4 for each concentration). The measured concentrations for each drug at each concentration was normalized and compared to the calculated concentration. Figure 5.7 represents the deviation of the measured concentration from the calculated concentration for each drug. The zero value on the y-axis indicates no deviation of the measured concentration to the calculated concentration. As the

concentration increases to 750 ng/mL and 1500 ng/mL, the accuracy of the measurement begins to decrease. Therefore fentanyl is no longer within ±20% error. The limit of quantitation for heroin, morphine, cocaine, codeine, and methadone is 2.9 ng/mL. The limit of quantitation for 6-acetylmorphine and fentanyl are 11.7 ng/mL for the SPE-IDMS method.

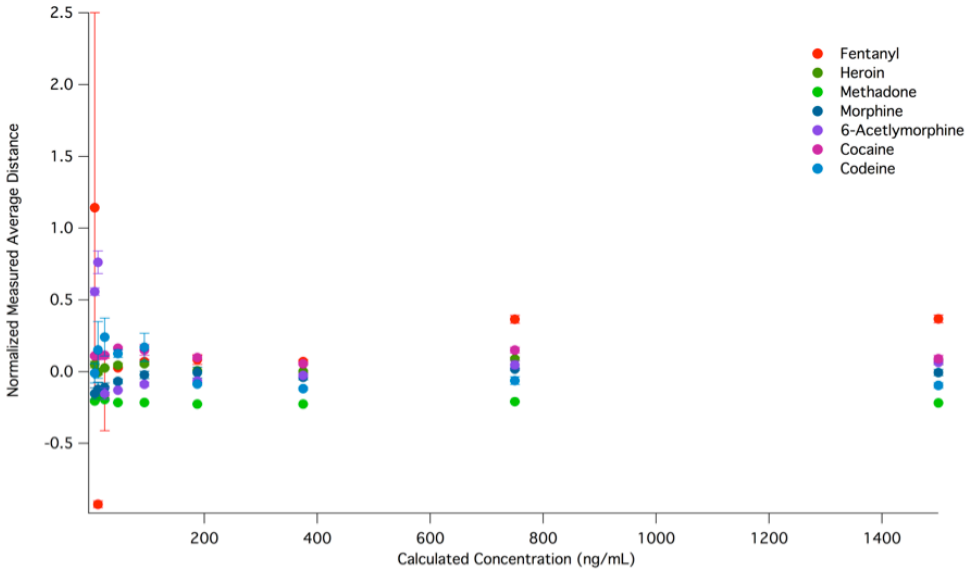


Figure 5.7. Limit of quantitation for SPE-IDMS technique

The limit of quantitation for the i-Spike method was determined by analyzing sample and spike concentrations of 200, 100, 50, 25, 12.5, 6.3, and 3.1 ng/mL (n=4 for each concentration). The measured concentration for each drug at each concentration was normalized and compared to the calculated concentration. Figure 5.8 represents the deviation of the measured concentration from the calculated concentration after normalization. The 95% confidence interval for each drug is within ±20% of the calculated concentration at a measured concentration of 3.1 ng/mL omitting codeine. The limit of quantitation for codeine with the i-Spike technique is 6.3 ng/mL.

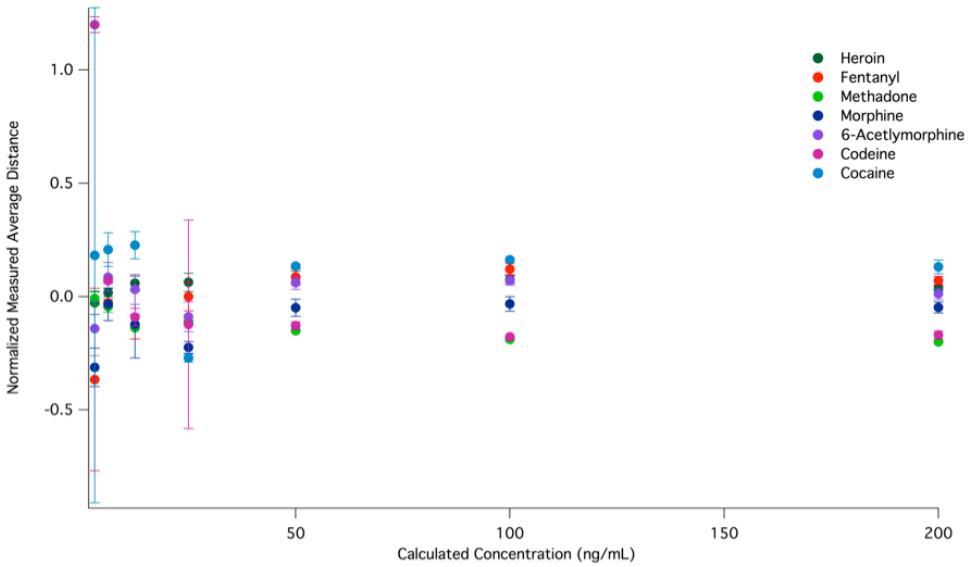


Figure 5.8. Limit of quantitation determination for i-Spike technique

To further decrease the limit of quantitation, the SPE-IDMS method was used to concentrate all seven drugs onto the SPE column. Samples were prepared at concentrations of 25, 12.5, 6.3, 3.1, 1.6, and 0.8 ng/mL of equilibrated naturally occurring drug and isotopically enriched drug at a 1:1 ratio (n=4 for each concentration). Each sample was concentrated onto the column up to a concentration of 25 ng/mL and compared. For example, a 25 ng/mL sample would be loaded onto the SPE column at a volume of 4.0 mL and a 12.5 ng/mL would be loaded onto the SPE column at a volume of 8.0 mL. Therefore, the same numbers of molecules are on both SPE columns. Figure 5.9 depicts the concentration of all seven drugs onto the SPE column.

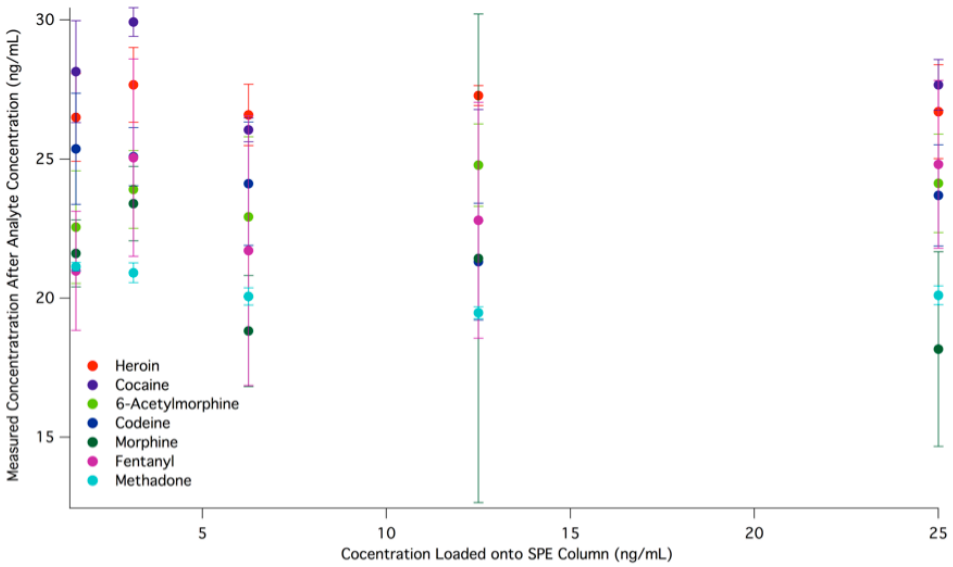


Figure 5.9. SPE-IDMS concentration of mixed drugs in synthetic urine samples

The measured concentrations are compared to the calculated concentration after SPE (25 ng/mL). All seven drugs can be quantitatively analyzed at a concentration of 0.8 ng/mL and still be within 20% relative standard deviation. Therefore, concentrating the analytes onto the SPE column is advantages allowing for the limit of quantitation to be lowered from 2.9 and 11.7 ng/mL to 0.8 ng/mL. This is inherently important for the quantitation of drugs in urine because of the rapid metabolism of the original drug administered.

5.3.2 Time stability analysis

The stability of the isotopically enriched analogues of all seven drugs pre-loaded onto the SPE column was assessed for validation of a field portable sample preparation method for the analysis of drugs in urine. The most stable preparation method was determined to be the addition of an individual frit above the commercially available SPE column from the analysis of glyphosate in water samples. Therefore, the same method was assessed with all seven drugs of abuse spiked onto the individual frit simultaneously in phosphate buffered saline solution. The samples were allowed to air dry and four SPE columns were

assessed at one week and two week time periods. Table 5.6 shows the IDMS analysis of seven drugs of abuse simultaneously using i-Spike after the designated isotope incubation period.

Table 5.6. Time stability analysis of seven drugs of abuse using ESI-TOF-MS

Drug	Time	Calc. Conc. (ng/mL)	Meas. Conc. (ng/mL)	Std. Dev.
Heroin				
	1 Week	40.000	54.450±0.587	1.101
	2 Weeks	40.000	57.755±0.350	0.657
6-Acetylmorphine				
	1 Week	40.000	47.335±0.905	1.698
	2 Weeks	40.000	54.163±0.573	1.074
Morphine				
	1 Week	40.000	51.729±0.567	1.064
	2 Weeks	40.000	54.393±0.813	1.526
Cocaine				
	1 Week	40.000	54.480±1.232	2.311
	2 Weeks	40.000	53.234±0.667	1.249
Codeine				
	1 Week	40.000	49.734±0.822	1.543
	2 Weeks	40.000	59.801±3.818	7.162
Fentanyl				
	1 Week	40.000	49.335±1.073	2.013
	2 Weeks	40.000	48.248±1.035	1.942
Methadone				
	1 Week	40.000	34.635±0.426	0.799
	2 Weeks	40.000	39.547±1.213	2.275

The calculated concentration for all analytes was 40.000 ng/mL and was subsequently compared to the measured concentration for each analyte after the designated time period. Every analyte except cocaine and fentanyl had an increase in concentration between one week and two weeks of analysis. The measured concentration after one week of incubation for heroin, 6-acetylmorphine, and morphine were 54.450±0.587, 47.335±0.905, and 51.729±0.567 ng/mL respectively. The concentrations of cocaine, codeine, fentanyl, and methadone were 54.480±1.232, 49.734±0.822, 49.335±1.073,

34.635±0.426 ng/mL respectively. All analytes had a positive bias except for methadone that had a negative bias in all analytical techniques. The positive bias is due to the irreversible binding of the isotopes onto the individual frit during incubation. The longer the isotope is loaded onto the frit before SPE, the higher the error is within the measurement. This may be due to activation of the column prior to analysis but also may be due to the loss of isotope in the various conditioning and wash steps in the SPE procedure.

The condition, wash, load, and elute steps of the i-Spike procedure were analyzed to determine if there was loss of analyte, isotope, or both during any part of the process. During the analysis, there was no indication or instrumental signal indicating any of the seven drugs in any part of the SPE process other than the elute step. It is possible that small amounts of isotope are lost in the conditioning steps that are below the limit of detection of the instrument. This would subsequently cause the signal of the isotope to be lower than what is expected causing the analyte to have a higher concentration than it actually is by using IDMS quantitation. If the isotope were lost during any of the steps after loading of the isotope, then the quantitation would be accurate due to equal loss of analyte and isotope after equilibration on the SPE column. To determine if there is loss of isotope in the condition steps, these steps could be eliminated starting the SPE procedure at the loading stage of the analyte.

Future work to increase the length of stability of the isotope on the SPE column could also include the preparation of the SPE packing in house instead of purchasing

commercially available SPE columns. During the column packing process, the isotopes can be massed and loaded onto the column with the packing. Each column would therefore be designated with their individualized masses of isotopes. This has the potential to eradicate the irreversible binding onto the column if influenced by tunneling or activation of the column.

5.4 Conclusions

Two accurate quantitative methods, SPE-IDMS and i-Spike have been developed for the analysis of heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl in synthetic urine. Both techniques are precise and rapid only taking 15 minutes for sample preparation and analysis. The limit of quantitation for the SPE-IDMS method was 2.9 ng/mL for heroin, morphine, cocaine, codeine, and methadone and 11.7 ng/mL for 6-acetylmorphinge and fentanyl. The i-Spike limit of quantitation is 6.3 ng/mL for codeine and 3.1 ng/mL for all other drugs. The limit of quantitation can be lowered to 0.8 ng/mL by concentrating the samples onto the SPE columns prior to elution. After SPE, the samples are analyzed directly using ESI-TOF-MS and does not require derivatization for increased ionization or separation using liquid chromatography.

The analysis of the dynamic range enables the determination of the maximum ratio of analyte to isotope that is still accurate and quantitative by IDMS. By analyzing ratios of analyte of analyte to isotope of 10:1, 2:1, 1:1, 1:2, and 1:10 it was determined that the most extreme ratio that maintains accuracy is 10:1 and 1:10. All five ratios, 1:10, 1:2, 1:1, 2:1, and 10:1, for each drug other then methadone are within a 20% relative standard deviation from the calculated concentration. The maximum analysis of the drugs is a 1:10

or 10:1 ratio of analyte to spike. Other applications of IDMS with ESI-TOF-MS have indicated ion suppression in the ionization process between the naturally occurring analyte and its isotopically enriched analogue. The ionization potential of the isotopically enriched analogue can effect the overall ionization of both the analyte and isotope in ESI. When the isotopically enriched analogue is only a one Dalton up-shift from the naturally occurring analyte little change occurs in the ionization potential creating ionization suppression of either the analyte or isotope. Each drug has been labeled with at least three deuterium's creating at minimum a three Dalton up-shift. Multiple deuteriums subsequently change the ionization potential of the isotopes. Therefore, when analyzing an unknown concentration of analyte, the ratio of analyte to isotope can deviate as much as a 1:10 or 10:1 ratio still enabling accurate and precise quantitation use ESI-TOF-MS. When using IDMS with isotopes of only one Dalton up-shift, ionization suppression can be corrected by using atmospheric pressure chemical ionization. Therefore, when analyzing an unknown sample ratio greater than 10:1 or 1:10, the results will be less accurate. The i-Spike method was also analyzed to determine the length of stability of the isotope when pre-loaded onto the SPE column. The analysis suggests that while using the individual frit to pre-load the isotope the isotope is only stable for less than one week. Measures can be taken to increase the stability of the isotope onto the column by packing in house SPE columns with the isotopes pre-loaded during the packing process and also eliminating the condition steps of the SPE procedure prior to the addition of the analytes.

These rapid methods have the potential to eliminate the need for immunological screening tests and elevate sample backlog. The samples analyzed in this chapter describe

the analysis in a synthetic urine system therefore not analyzing morphine-3-glucuronide and morphine-6-glucuronide. Also, the metabolites of cocaine and other contaminants were not investigated, but with the structural similarities of the metabolites and the resolving power of the TOF-MS, these methods can be extended to numerous analytes. The utilization of IDMS for quantitation not only allows accurate quantitation of each drug but it has the capabilities of tracking the metabolism of the drugs and their metabolites for a more accurate quantitation method. These methods can screen for numerous drugs, metabolites, and common contaminants and are also quantitatively accurate by employing IDMS.

Chapter 6

Conclusions

6.1 Mathematical determination for molecular isotope dilution mass spectrometry

Traditional IDMS equations were modified for the accurate quantitation of molecular compounds. The traditional IDMS equations are utilized for elemental analysis, therefore taking into account only the natural abundances of the element itself. When assessing molecules for IDMS quantitation, the elements other than the isotopically enriched element also contribute to the naturally occurring probability of a contribution of analyte to spike and spike to analyte. Therefore, statistical analysis was completed for the probabilities of B_{sp}, A_{sp}, B, and A for any molecular compound. Table 2.2 illustrates the IDMS constants for glyphosate, methylphosphonic acid, heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl.

The isotopic enrichment directly contributes the constants B_{sp}, A_{sp}, B, and A for any molecular compound. Glyphosate is an example of a compound that has a single label on a carbon atom within the molecule. This single label causes only a one Dalton up shift in the mass spectra for glyphosate-2-^{13}C compared to the analyte. Therefore, there is a greater probability of contribution from other natural abundant forms other elements in the compound such as oxygen, hydrogen, nitrogen, and carbon. The more isotopic enrichments in a given molecule, the lower the probability that the analyte will naturally contribute to the spike peak. This is seen in A_{sp}, where glyphosate has a single enrichment

and a value of 0.00951 and methylphosphonic acid, with four enrichments, has a value of 7.936×10^{-8}. This is also seen in the illicit drug analysis with deuterium labeled analytes. Heroin has nine deuterated hydrogen atoms and an A_{sp} value of 3.988×10^{-16} while 6-acetylmorphine has only three deuterated atoms hydrogen and an A_{sp} value of 6.381×10^{-6}.

With the determination of these constants for the molecules being analyzed, the IDMS equation can be extended to the analysis of complex molecules and is not limited to the analysis of elemental species. The IDMS equation now takes into account contributions of standard purity, analyte to spike contributions, and spike to analyte contributions. These equations have been utilized in the analysis of environmental and biological samples. The statistical analysis of the contributions of the analyte to the spike and spike to analyte enables accurate quantitation of molecular compounds using IDMS. These equations have the ability to be implemented into mathematical software that could compute the IDMS constants for any molecule. A computational approach would simplify IDMS analysis for molecular compounds enabling it to be a standardized accurate quantitative method not just for elemental analysis but also molecular analysis.

6.2 Quantitation of phosphate based environmental molecules in water samples

EPA method 6800 has been extended to the analysis of glyphosate in drinking water samples. The dynamic range analysis of a standardized analyte is required for the proper determination of the ratio range of the analyte. The analysis of a variety of ratios of analyte to isotope using ESI-TOF-MS presented ionization suppression within the sample. When an isotopically labeled analyte is only enriched with a single isotope,

suppression occurs with the peak that is of the lower abundance in the mass spectra. This ion suppression was compensated for using an APCI-Q-TOF-MS. With APCI, the constant electron source from the corona discharge needle decreases ion suppression. The ideal ratio between glyphosate and glyphosate-2-^{13}C is 1:1 but a ratio extreme of 10:1 and 1:10 can be analyzed while still maintaining accuracy and precision.

The optimized ESI-TOF-MS and APCI-Q-TOF-MS methods were utilized for the validation of the two newly developed SPE methods. The SPE-IDMS and i-Spike techniques are comparable to the traditional IDMS quantitation of both glyphosate in drinking water. Once the samples are spiked with the isotopically enriched spike for the SPE-IDMS analysis, sample loss and retention, as well as instrument fluctuations and drift do not adversely affect the quantitation. The i-Spike technique has the potential to analyze drinking water samples, de novo, with the isotopically enriched spike previously loaded onto the column. With the utilization of an individual frit, the isotope is stable on the SPE column for two weeks prior to analysis.

The limit of quantitation for both SPE-IDMS and i-Spike techniques when the analyte is concentrated onto the SPE column is 0.400 ppm for glyphosate. The detection limit for glyphosate after column concentration is lower then the maximum containment level of 0.7 ppm set by the National Primary Drinking Water Regulations from the USEPA[11]. These techniques have the potential to be employed for a rapid and reliable analytical method of glyphosate and other phosphonic acid containing compounds in water samples that does not require time-consuming derivatization or liquid chromatography separation

prior to analysis on a TOF-MS. The methods described here are amenable for analyses of a variety of drinking water analytes. Direct sample equilibration SPE-IDMS or by i-Spike, reduced the biases caused by recovery, calibration and ionization differences without the need of calibration curves and eliminates derivatization. By adapting direct IDMS measurements of glyphosate, future automation and similarly accurate applications for analysis of other molecules, such as pesticides, toxins and toxicants in potable water can be foreseen.

6.3 Quantitation of phosphate based nerve agents in environmental matrices

The SPE-IDMS and i-Spike methods developed for the analysis of glyphosate in drinking water samples have successfully been extended to the analysis of methylphosphonic acid in water samples. Analysis was completed on an APCI-Q-TOF-MS with a limit of quantitation of 0.25 ppm. The SPE-IDMS and i-Spike methods were compared and validated against traditional IDMS analysis to determine validity of the methods. Traditional IDMS had a measured concentration of 0.942±0.008 ppm (n=16) compared to a normalized calculated concentration of 1.00 ppm. SPE-IDMS and i-Spike methodologies had measured concentrations of 0.862±0.045 (n=16) and 0.846±0.011 ppm (n=16) respectively. This data is well within the USEPA standard of a 20% relative standard deviation for a given sample. The slight bias of the measurements may be explained by the difference in retention between the glyphosate and the methylphosphonic acid in the SPE column with the established methods. Another potential explanation of the slightly low bias within the methylphosphonic acid measurement as compared to glyphosate is the ionization differences between ^{13}C and ^{2}H.

It has been noted in the literature that ^{2}H labeled analogues when compared to ^{13}C labeled analogues have a slight negative bias. This may explain why glyphosate analysis did not indicate a bias since it is ^{13}C labeled. Methylphosphonic acid, on the other hand, has not only a ^{13}C isotopic label but also three ^{2}H labels. This may be causing the bias within measurement and must be investigated further.

The limit of quantitation for the SPE columns for both SPE-IDMS and i-Spike methods were investigated to further validate the sample preparation methods. SPE-IDMS had a limit of quantitation of 0.500 ppm and 0.200 ppm respectively. To further decrease the limit of quantitation for methylphosphonic acid, the analyte was concentrated onto the SPE column. When concentrating methylphosphonic acid, the limit of quantitation is extended to 0.031 ppm.

To determine the applicability of the SPE-IDMS and i-Spike methods for the analysis of phosphonic acid based nerve agents and environmental samples, glyphosate and methylphosphonic acid were assessed simultaneously and quantified. Both glyphosate and methylphosphonic acid measurements were precise with measured concentrations of 5.770±0.113 ppm (n=4) and 0.880±0.059 ppm (n=4) respectively. The calculated concentration for glyphosate was 6.00 ppm and 1.00 ppm for methylphosphonic acid. This preliminary data suggests that the two newly developed SPE techniques have the ability to assess samples containing multiple phosphonic acid containing compounds.

6.4 Alternative method for the quantitation of illicit drugs, metabolites, and contaminants in urine correcting for metabolism

Two accurate quantitative methods, SPE-IDMS and i-Spike have been developed for the analysis of heroin, 6-acetylmorphine, morphine, cocaine, codeine, methadone, and fentanyl in synthetic urine. Both techniques are accurate, precise, and rapid only taking 15 minutes for sample preparation and analysis. The limits of quantitation for the SPE-IDMS method was 2.9 ng/mL for heroin, morphine, cocaine, codeine, and methadone and 11.7 ng/mL for 6-acetylmorphine and fentanyl. The i-Spike limit of quantitation was 6.3 ng/mL for codeine and 3.1 ng/mL for all other drugs. The limit of quantitation can be lowered to 0.8 ng/mL by concentrating the samples onto the SPE columns prior to elution. After SPE, the samples are analyzed directly using ESI-TOF-MS and does not require derivatization for increased ionization or separation using liquid chromatography.

The analysis of the dynamic range enables the determination of the maximum ratio of analyte to isotope that is still accurate and quantitative by IDMS. By analyzing ratios analyte to isotope of 10:1, 2:1, 1:1, 1:2, and 1:10 it was determined that the most extreme ratio that maintains accuracy is 10:1 and 1:10. Therefore, when analyzing an unknown sample a ratio of greater than 10:1 or 1:10 will produce results that are less accurate. The i-Spike method was also analyzed to determine the length of stability of the isotope when pre-loaded onto the SPE column. The analysis suggests that while using the individual frit for pre-loading the isotope is only stable for one week. Measures can be taken to increase the stability of the isotope onto the column by packing in house SPE columns

with the isotopes pre-loaded during the packing process and also eliminating the conditioning steps of the SPE procedure prior to the addition of the analytes.

These rapid methods have the potential to eliminate the need for immunological screening tests and elevate sample backlog. The samples analyzed were in a synthetic urine system therefore not analyzing for morphine-3-glucuronide and morphine-6-glucuronide. Although the metabolites of heroin were investigated, the metabolites of cocaine and other contaminants were not investigated, but with the structural similarities of the metabolites and the resolving power of the TOF-MS, these methods can be extended to numerous analytes. The utilization of IDMS for quantitation not only allows accurate quantitation of each drug but it has the capabilities of tracking the metabolism of the drugs and their metabolites for a more accurate quantitation method. These methods not only can screen for numerous drugs, metabolites, and common contaminants but it is also quantitatively accurate by employing IDMS.

Chapter 7 References

1. Borjesson, E.; Torstensson, L., New methods for determination of glyphosate and (aminomethyl)phosphonic acid in water and soil. *J. Chromatogr. A* **2000,** *886* (1-2), 207-16.
2. Ibanez, M.; Pozo, O. J.; Sancho, J. V.; Lopez, F. J.; Hernandez, F., Residue determination of glyphosate, glufosinate and aminomethylphosphonic acid in water and soil samples by liquid chromatography coupled to electrospray tandem mass spectrometry. *J. Chromatogr. A* **2005,** *1081* (2), 145-155.
3. Ibanez, M.; Pozo, O. J.; Sancho, J. V.; Lopez, F. J.; Hernandez, F., Re-evaluation of glyphosate determination in water by liquid chromatography coupled to electrospray tandem mass spectrometry. *J. Chromatogr. A* **2006,** *1134* (1-2), 51-55.
4. Patsias, J.; Papadopoulou, A.; Papadopoulou-Mourkidou, E., Automated trace level determination of glyphosate and aminomethyl phosphonic acid in water by on-line anion-exchange solid-phase extraction followed by cation-exchange liquid chromatography and post-column derivatization. *J. Chromatogr. A* **2001,** *932* (1-2), 83-90.
5.Popp, M.; Hann, S.; Mentler, A.; Fuerhacker, M.; Stingeder, G.; Koellensperger, G., Determination of glyphosate and AMPA in surface and waste water using high-performance ion chromatography coupled to inductively coupled plasma dynamic reaction cell mass spectrometry (HPIC-ICP-DRC-MS). *Anal. Bioanal. Chem.* **2008,** *391* (2), 695-9.
6. Paterson, S.; Lintzeris, N.; Mitchell, T. B.; Cordero, R.; Nestor, L.; Strang, J., Validation of techniques to detect illicit heroin use in patients prescribed pharmaceutical heroin for the management of opioid dependence. *Addiction* **2005,** *100* (12), 1832-9.
7. Tetsuya, I.; Ishijima, C.; Ohashi, A.; Okada, H.; and Ohashi, K., Solid Phase Extraction of Phosphorus-containing Amino Acid-type Herbicides and Their Metabolites from Human Blood with Titania for Determination by Capillary Electrophoresis. *Anal. Sci.* **2007,** *23*, 755-758.
8. Hori, Y.; Shimada, K.; and Hirose, Y., Determination of the Herbicide Glyphosate and its Metabolite in Biological Specimens by Gas Chromatography-Mass Spectrometry. A Case of Poisoning by Roundup Herbicide. *J. Anal. Tox.* **2003,** *27*, 162-166.
9. Guo, Z. X.; Cai, Q.; Yang, Z., Determination of glyphosate and phosphate in water by ion chromatography--inductively coupled plasma mass spectrometry detection. *J. Chromatogr. A* **2005,** *1100* (2), 160-7.
10. Mink, P. J.; Mandel, J. S.; Lundin, J. I.; Sceurman, B. K., Epidemiologic studies of glyphosate and non-cancer health outcomes: A review. *Regul. Toxicol. Pharmacol.* **2011**.
11. Agency, U. S. E. P., National Primary Drinking Water Regulations 2009.

12. Cox, C., Glyphosate (Roundup). *J. Pest. Reform.* **1998,** *18*, 3-17.
13. Acquavella, J. F.; Alexander, B. H.; Mandel, J. S.; Gustin, C.; Baker, B.; Chapman, P.; Bleeke, M., Glyphosate biomonitoring for farmers and their families: results from the Farm Family Exposure Study. *Environ. Health. Perspect.* **2004,** *112* (3), 321-6.
14. Takahashi, M.; Horie, M.; Aoba, N., Analysis of glyphosate and its metabolite, aminomethylphosphonic acid, in agricultural products by HPLC. *Shokuhin Eiseigaku Zasshi* **2001,** *42*, 304-308.
15. Gasnier, C.; Dumont, C.; Benachour, N.; Clair, E.; Chagnon, M. C.; Seralini, G. E., Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology* **2009,** *262* (3), 184-91.
16. Dimitrakopoulos, I. K.; Thomaidis, N. S.; Megoulas, N. C.; Koupparis, M. A., Effect of suppressor current intensity on the determination of glyphosate and aminomethylphosphonic acid by suppressed conductivity ion chromatography. *J. Chromatogr. A* **2010,** *1217* (22), 3619-27.
17. Kudzin, Z. H.; Gralak, D. K.; Andrijewski, G.; Drabowicz, J.; Luczak, J., Simultaneous analysis of biologically active aminoalkanephosphonic acids. *J. Chromatogr. A* **2003,** *998* (1-2), 183-99.
18. Kudzin, Z. H.; Gralak, D. K.; Drabowicz, J.; Luczak, J., Novel approach for the simultaneous analysis of glyphosate and its metabolites. *J. Chromatogr. A* **2002,** *947* (1), 129-141.
19. Motojyuku, M.; Saito, T.; Akieda, K.; Otsuka, H.; Yamamoto, I.; Inokuchi, S., Determination of glyphosate, glyphosate metabolites, and glufosinate in human serum by gas chromatography-mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2008,** *875* (2), 509-14.
20. Stalikas, C. D.; Pilidis, G. A., Development of a method for the simultaneous determination of phosphoric and amino acid group containing pesticides by gas chromatography with mass-selective detection optimization of the derivatization procedure using an experimental design approach. *J. Chromatogr. A* **2000,** *872* (1-2), 215-25.
21. Ghanem, A.; Bados, P.; Kerhoas, L.; Dubroca, J.; Einhorn, J., Glyphosate and AMPA analysis in sewage sludge by LC-ESI-MS/MS after FMOC derivatization on strong anion-exchange resin as solid support. *Anal. Chem.* **2007,** *79* (10), 3794-801.
22. Hernandez, F.; Sancho, J. V.; Pozo, O.; Lara, A.; Pitarch, E., Rapid direct determination of pesticides and metabolites in environmental water samples at sub-microg/l level by on-line solid-phase extraction-liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A* **2001,** *939* (1-2), 1-11.
23. Lee, E. A.; Zimmerman, L. R.; Bhullar, B. S.; Thurman, E. M., Linker-assisted immunoassay and liquid chromatography/mass spectrometry for the analysis of glyphosate. *Anal. Chem.* **2002,** *74* (19), 4937-43.
24. Sancho, J. V.; Pozo, O. J.; Hernandez, F., Liquid chromatography and tandem mass spectrometry: a powerful approach for the sensitive and rapid multiclass

determination of pesticides and transformation products in water. *Analyst* **2004,** *129* (1), 38-44.
25. Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction. Washington, DC, 1993.
26. Ellison, D. H., *Handbook of Chemical and Biological Warfare Agents* Second Edition ed.; CRC Press: Boca Raton, 2008; p 762.
27. Mawhinney, D.B.; Fraser, R.; Silva, S.S.; Pavlopoulos, A.J., and Kobelski, R.J., The determination of organophosphonate nerve agent metabolites in human urine by hydrophilic interaction liquid chromatography tandem mass spectrometry *J. Chromatogr. B* **2007,** *852*, 9.
28. Worek, F.; Thiermann, H.; Szinicz, L., Diagnostic Aspects of Organophosaphate Poisoning *Toxicology* **2005,** *214,* 8.
29. Steiner, W. E.; Harden, C. S.; Hong, F.; Klopsch, S. J.; Hill, H. H., Jr.; McHugh, V. M., Detection of aqueous phase chemical warfare agent degradation products by negative mode ion mobility time-of-flight mass spectrometry [IM(tof)MS]. *J. Am. Soc. Mass Spectrom.* **2006,** *17* (2), 241-5.
30. Ciner, F. L.; McCord, C. E.; Plunkett, R. W., Jr.; Martin, M. F.; Croley, T. R., Isotope dilution LC/MS/MS for the detection of nerve agent exposure in urine. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2007,** *846* (1-2), 42-50.
31. Lee, H. S.; Basheer, C.; Lee, H. K., Determination of trace level chemical warfare agents in water and slurry samples using hollow fibre-protected liquid-phase microextraction followed by gas chromatography-mass spectrometry. *J. Chromatogr. A* **2006,** *1124* (1-2), 91-6.
32. Saradhi, V.; Reddy, T.; and Vairamani, M., Ion-pair solid-phase extraction and gas chromatography-mass spectrometric determination of acidic hydrolysis products of chemical warfare agents from aqueous samples *J. Chromatogr. A* **2006,** *1129*, 5.
33. Kanamori-Kataoka, Y., Mass Spectrometric Stategy for the Determination of Natural and Synthetic Organic Toxins *J. Health Sci.* **2005,** *51* (5), 7.
34. Asbury, G.R.; Siems, W.F.; and Hill, H.H., Separation and identification of some chemical warfare degradation products using electrospray high resolution ion mobility spectrometry with mass selected detection *Analytica Chimica Acta* **2000,** *404*, 11.
35. Sega, G.A.; and Griest, W.H.; Analysis of methyphosphonic acid, ethyl methylphosphonic acid, and isopropyl methylphosphonic acid at low microgram per liter levels in ground water *J. Chromatogr. A* **1997,** *790,* 10
36. Wang, J.; Collins, G.E.; and Mulchandani, A., Measurements of chemical warfare agent degradation products using an electrophoresis microchip with contactless conductivity detector *Anal. Chem.* **2002,** *74,* 5
37. Read, R. W.; Black, R. M., Rapid screening procedures for the hydrolysis products of chemical warfare agents using positive and negative ion liquid chromatography-mass spectrometry with atmospheric pressure chemical ionisation. *J. Chromatogr. A* **1999,** *862* (2), 169-77.

38. Piao, H.; Marx, R. B.; Schneider, S.; Irvine, D. A.; Staton, J., Analysis of VX nerve agent hydrolysis products in wastewater effluents by ion chromatography with amperometric and conductivity detection. *J. Chromatogr. A* **2005,** *1089* (1-2), 65-71.
39. Riches, J.; Morton, I.; Read, R. W.; Black, R. M., The trace analysis of alkyl alkylphosphonic acids in urine using gas chromatography-ion trap negative ion tandem mass spectrometry. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* **2005,** *816* (1-2), 251-8.
40. D'Agostino, P. A.; Hancock, J. R.; Chenier, C. L.; Lepage, C. R., Liquid chromatography electrospray tandem mass spectrometric and desorption electrospray ionization tandem mass spectrometric analysis of chemical warfare agents in office media typically collected during a forensic investigation. *J. Chromatogr. A* **2006,** *1110* (1-2), 86-94.
41. Liu, Q.; Hu, X.; Xie, J., Determination of nerve agent degradation products in environmental samples by liquid chromatography-time-of-flight mass spectrometry with electrospray ionization. *Anal. Chim. Acta* **2004,** *512*, 93-101.
42. U.S. EPA Method 6800 in Test Methods for Evaluative Solid Water, Physical/Chemical Methods SW846, Update IVA. Agency, U. S. E. P., Ed. Washington, DC, **2008**.
43. Services, U. S. D. o. H. a. H. *Results from the 2009 National Survey on Drug Use and Health: Volume I. Summary of National Findings* **2010**.
44. (DAWN), D. A. W. N. *Highlights of the 2009 Drug Abuse Warning Network (DAWN) Findings on Drug-Related Emergency Department Visits* **2010**.
45. Services, U. S. D. o. H. a. H. *Drug Abuse Warning Network, 2007: National Estimates of Drug-Related Emergency Department Visits* **2007**.
46. (DAWN), D. A. W. N. *Opiate-related drug misuse deaths in six states: 2003*; **2006**.
47. Al-Asmari, A. I.; Anderson, R. A., Method for quantification of opioids and their metabolites in autopsy blood by liquid chromatography-tandem mass spectrometry. *J. Anal. Toxicol.* **2007,** *31* (7), 394-408.
48. Rook, E. J.; Hillebrand, M. J.; Rosing, H.; van Ree, J. M.; Beijnen, J. H., The quantitative analysis of heroin, methadone and their metabolites and the simultaneous detection of cocaine, acetylcodeine and their metabolites in human plasma by high-performance liquid chromatography coupled with tandem mass spectrometry. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* **2005,** *824* (1-2), 213-21.
49. Rook, E. J.; Huitema, A. D.; van den Brink, W.; van Ree, J. M.; Beijnen, J. H., Pharmacokinetics and pharmacokinetic variability of heroin and its metabolites: review of the literature. *Curr. Clin. Pharmacol.* **2006,** *1* (1), 109-18.
50. Coleman, M. D., *Appendix B metabolism of major illicit drugs* John Wiley & Sons 2005.

51. McDonough, P. C.; Levine, B.; Vorce, S.; Jufer, R. A.; Fowler, D., The detection of hydromorphone in urine specimens with high morphine concentrations. *J. Forensic Sci.* **2008,** *53* (3), 752-4.
52. Jones, A. W.; Holmgren, A.; Kugelberg, F. C., Driving under the influence of opiates: concentration relationships between morphine, codeine, 6-acetyl morphine, and ethyl morphine in blood. *J. Anal. Toxicol.* **2008,** *32* (4), 265-72.
53. Rook, E. J.; Huitema, A. D.; van den Brink, W.; Hillebrand, M. J.; van Ree, J. M.; Beijnen, J. H., Screening for illicit heroin use in patients in a heroin-assisted treatment program. *J. Anal. Toxicol.* **2006,** *30* (6), 390-4.
54. Avella, J.; Lehrer, M., Assesing free and total and free morphine following heroin overdose when complicated by the presence of toxic amitriptyline levels *J. Anal. Toxicol.* **2007,** *31* (3).
55. DiPietro, N.; Fiorini, I.; Manera, C.; Orlando, C.; Palombo, F.; Saccomanni, G.; Salotti, V.; Macchia, M., Use of capillary electrophoresis and polu(ethylene oxide) as the coating agent for the determination of substances related to heroin addiction and treatment. *J. Anal. Toxicol.* **2006,** *30* (4).
56. Cardero, S. P., Comparison of the various opiate alkaloid contaminants and their metabolites found in illicit heroin with 6-monoacetyl morphine as indicators of heroin ingestion. *J. Anal. Toxicol.* **2006,** *30* (7).
57. Staub, C.; Marset, M.; Mino, A.; Mangin, P., Detection of acetylcodeine in urine as an indicator of illicit heroin use: method validation and results of a pilot study. *Clin. Chem.* **2001,** *47* (2), 301-7.
58. Wong, S. C.; Curtis, J. A.; Wingert, W. E., Concurrent detection of heroin, fentanyl, and xylazine in seven drug-related deaths reported from the Philadelphia Medical Examiner's Office *J. Forensic Sci.* **2008,** *53* (2), 495-498.
59. Hull, M. J.; Juhascik, M.; Mazur, F.; Flomenbaum, M. A.; Behonic, G. S., Fatalities associated with fentanyl and co-administered cocaine or opiates *J. Forensic Sci.* **2007,** *52* (6), 1383-88.
60. Shields, L. B.; Hunsaker, D. M.; Hunsaker, J. C., 3rd, Schizophrenia and suicide: a 10-year review of Kentucky medical examiner cases. *J. Forensic Sci.* **2007,** *52* (4), 930-7.
61. Mclachlan-Troup, N.; Taylor, G. W.; Trathen, B. C., Diamorphine treatment for opiate dependance: putative markers of concomitant heroin misuse. *Addiction Biology* **2001,** *6*, 223-231.
62. Cheong, J. C.; Suh, S. I.; Ko, B. J.; Kim, J. Y.; In, M. K.; Cheong, W. J., Gass chromatography-mass spectrometric method for the screening and quantification of illicit drugs and their metabolites in human urine using solid-phase extraction and trimethylsilyl derivatization. *J. Sep. Sci.* **2010,** *33*, 1767-1778.
63. Gustavsson, E.; Andersson, M.; Stephanson, N.; Beck, O., Validation of direct injection electrospray LC-MS/MS for confirmation of opiates in urine drug testing. *J. Mass Spectrom.* **2007,** *42* (7), 881-9.

64. Pichini, S.; Altieri, I.; Pellegrini, M.; Zuccaro, P.; Pacifici, R., The role of liquid chromatography-mass spectrometry in the determination of heroin and related opioids in biological fluids. *Mass Spectrom. Rev.* **1999,** *18* (2), 119-30.
65. Wolff, K.; Farrell, M.; Marsden, J.; Monteiro, M. G.; Ali, R.; Welch, S.; Strang, J., A review of biological indicators of illicit drug use, practical considerations and clinical usefulness. *Addiction* **1999,** *94* (9), 1279-98.
66. Crandall, C.S.; Kerrigan, S.; Blau, R.L.; Lavalley, J.; Zumwalt, R.; McKinney, P. E., The influence of site of collection on postmortem morphine concentrations in heroin overdose victims. *J. Forensic Sci.* **2006,** *51* (2), 413-20.
67. Cristoni, S.; Cantu, M.; Bernardi, L.R.; Gerthoux, P.; Mocarelli, P.; Brambilla, M.; Gonella, E.; Guidugli, F., Surface-activated chemical ionization ion trap mass spectrometry in the analysis of drugs in dilute urine samples. Part II: analysis of morphine and other street drugs. *J. Mass Spectrom.* **2005,** *40* (12), 1609-17.
68. Couchman, L.; Morgan, P.E., LC-MS in analytical toxicology: some practical considerations. *Biomed. Chromatogr.* **2011,** *25* (1-2), 100-23.
69. Gergov, M.; Ojanpera, I.; Vuori, E., Simultaneous screening for 238 drugs in blood by liquid chromatogrphy-ionspray tandem mass spectrometry with multiple-reaction monitoring *J.Chromatogr. B* **2003,** *795*, 41-53.
70. Shields, L. B.; Hunsaker III, J. C.; Corey, T. S.; Ward, M. K.; Stewart, D., Methadone toxicity fatalities: A review of medical examiner cases in a large metropolitan area. *J. Forensic Sci.* **2007,** *52* (6), 1389-1395.
71. Dams, R.; Murphy, C. M.; Lambert, W. E.; Huestis, M. A., Urine drugs testing for opioides, cocatine, and metabilites by direct injection liquid chromatography/ tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003,** *17*, 1665-1670.
72. Moeller, M. R.; Steinmeyer, S.; Kraemer, T., Determination of drugs of abuse in blood *J. Chromatogr. B* **1998,** *713*, 91-109.
73. Feng, J.; Wang, L.; Dai, I.; Harmon, T.; Bernert, J. T., Simultaneous determination of multiple drugs of abuse and relevant metabolites in urine by LC-MS-MS. *J. Anal. Toxicol.* **2007,** *31* (7), 359-68.
74. Cardenas, S.; Gallego, M.; Valcarcel, M., An automated preconcentration-derivatization system for the determination of cocaine and its metabolites in urine and illicit cocaine samples by gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **1996,** *10* (6), 631-6.
75.Cardenas, S.; Gallego, M.; Valcarcel, M., Rapid solid-phase extraction/ derivatization system for sample preparation and gas chromatographic/mass spectrometric determination of drugs in human urine. *Rapid Commun. Mass Spectrom.* **1997,** *11* (3), 298-306.
76. Nunez, O.; Moyano, E.; Galceran, M. T., Time-of-flight high resoluton versus triple quadrupole tandem mass spectrometry for the analysis of quaternary ammonium herbicides in drinking water *Anal. Chim. Acta* **2004,** *525*, 183-190.
77. Huck, C. W.; Bonn, G. K., Recent developments in polymer-based sorbents for solid-phase extraction. *J. Chromatogr. A* **2000,** *885* (1-2), 51-72.

78. Hennion, M. C., Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography. *J. Chromatogr. A* **1999,** *856* (1-2), 3-54.
79. Poole, C. F., New trends in solid-phase extraction. *Trends in Anal. Chem.* **2003,** *22* (6), 362-373.
80. Hill, S. J.; Pitts, L. J.; Fisher, A. S., High-performance liquid chromatography-isotope dilution inductively coupled plasma mass spectrometry for speciation studies: an overview. *Trends in Anal. Chem.* **2000,** *19*, 120-126.
81. Rodriguez-Gonzalez, P.; Marchante-Gayon, J. M.; Garcia Alonso, J. I.; Sanz-Medel, A., Isotope dilution analysis fro elemental speciation: A tutorial review. *Spectrochimica Acta Part B* **2005,** *60*, 151-207.
82. Rosen, A. L.; Hieftje, G. M., Inductively coupled plasma mass spectrometry and electrospray mass spectrometry for speciation analysis: application and instrumentation. *Spectrochimica Acta Part B* **2004,** *59*, 135-146.
83. Hinojosa Reyes, L.; Marchante-Gayon, J. M.; Alonso, J. I.; Sanz-Medel, A., Quantitative speciation of selenium in human serum by affinity chromatography coupled to post-column isotope dilution analysis ICP-MS. *J. Anal. At. Spectrom* **2003,** *18*, 1210-1216.
84. Meija, J.; Mester, Z., Paradigms in isotope dilution mass spectrometry for elemental speciation analysis. *Anal. Chim. Acta* **2008,** *607* (2), 115-25.
85. Pedrero, Z.; Encinar, J. R.; Madrid, Y.; Camara, C., Identification of selenium species in selenium-enriched Lens esculenta plants by using two-dimensional liquid chromatography-inductively coupled plasma mass spectrometry and [77Se]selenomethionine selenium oxide spikes. *J. Chromatogr. A* **2007,** *1139* (2), 247-53.
86. Yang, L.; Sturgeon, R. E.; McSheehy, S.; Mester, Z., Comparison of extration methods for quantitation of methionine and selenomethionine in yeast by species specific isotope dilution gas chromatography-mass spectrometry. *J. Chromatogr. A* **2004,** *1055*, 177-184.
87. Loo, J.A., Electrospray ionization mass spectrometry: a technology for studying noncovalent macromolecular complexes *International J. Mass Spectrom.* **2000,** *200*, 175-186.
88. Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M., Electrospray ionization for mass spectrometry of large biomolecules. *Science* **1989,** *246* (4926), 64-71.
89. Smith, J. N.; Flagan, R. C.; Beauchamp, J. L., Droplet evaporation and discharge dynmanis in electrospray ionization. *J. Phys. Chem.* **2002,** *106*, 9957-9967.
90. Cech, N. B.; Enke, C. G., Practical implications of some recent studies in electrospray ionization fundamentals. *Mass. Spectrom. Rev.* **2001,** *20* (6), 362-87.
91. Andrade, F. J.; Shelley, J. T.; Wetzel, W. C.; Webb, M. R.; Gamez, G.; Ray, S. J.; Hieftje, G. M., Atmospheric pressure chemical ionization source. 1. Ionization of compounds in the gas phase. *Anal. Chem.* **2008,** *80* (8), 2646-53.

92. Hoffmann, E.; Stroobant, V., *Mass spectrometry principles and applications* Third Edition ed.; John Wiley and Sons, Ltd: **2007**.
93. Technologies, A., Agilent G3250AA LC/MSD TOF System Concepts Guide. In *Agilent Technologies*, Fourth Edition ed.; Santa Clara, CA, **2005**.
94. Schilling, A., TOF Theory In *PowerPoint Presentation* Agilent Technologies May 7, **2007**
95. Skoog, D. A.; Holler, F. J.; Crouch, S. R., *Principles of instrumental analysis*. 6th Edition ed.; Thomson Higher Education: **2007**.
96. Busto, M. E.; Montes-Bayon, M.; Sanz-Medel, A., Accurate determination of human serum transferrin isoforms: Exploring metal-specific isotope dilution analysis as a quantitative proteomic tool. *Anal Chem* **2006,** *78* (24), 8218-26.
97. Harrington, C. F.; Vidler, D. S.; Watts, M. J.; Hall, J. F., Potential for using isotopically altered metalloproteins in species-specific isotope dilution analysis of proteins by HPLC coupled to inductively coupled plasma mass spectrometry. *Anal. Chem.* **2005,** *77* (13), 4034-41.
98. Meija, J.; Caruso, J. A., Selenium and sulfur trichalcogenides from the chalcogenide exchange reaction. *Inorg. Chem.* **2004,** *43* (23), 7486-92.
99. Itoh, N.; Numata, M.; Aoyagi, Y.; Yarita, T., Comparison of the behavior of 13C- and deuterium-labeled polycyclic aromatic hydrocarbons in analyses by isotope dilution mass spectrometry in combination with pressurized liquid extraction. *J. Chromatogr. A* **2007,** *1138* (1-2), 26-31.
100. McCoy, L. F.; Scholl, P. F.; Schleicher, R. L.; Groopman, J. D.; Powers, C. D.; Pfeiffer, C. M., Analysis of aflatoxin B1-lysine adduct in serum using isotope-dilution liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005,** *19* (16), 2203-10.
101. Stalikas, C. D.; Pilidis, G. A., Development of a method for the simultaneous determination of phosphoric and amino acid group containing pesticides by gas chromatography with mass-selective detection optimization of the derivatization procedure using an experimental design approach. *J. Chromatogr. A* **2000,** *872* (1-2), 215-25.
102. Kudzin, Z. H.; Gralak, D. K.; Andrijewski, G.; Drabowicz, J.; Luczak, J., Simultaneous analysis of biologically active aminoalkanephosphonic acids. *J. Chromatogr. A* **2003,** *998* (1-2), 183-99.
103. Motojyuku, M.; Saito, T.; Akieda, K.; Otsuka, H.; Yamamoto, I.; Inokuchi, S., Determination of glyphosate, glyphosate metabolites, and glufosinate in human serum by gas chromatography-mass spectrometry. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* **2008,** *875* (2), 509-14.
104. Lee, E. A.; Zimmerman, L. R.; Bhullar, B. S.; Thurman, E. M., Linker-assisted immunoassay and liquid chromatography/mass spectrometry for the analysis of glyphosate. *Anal. Chem.* **2002,** *74* (19), 4937-43.
105. Hernandez, F.; Sancho, J. V.; Pozo, O.; Lara, A.; Pitarch, E., Rapid direct determination of pesticides and metabolites in environmental water samples at

sub-microg/l level by on-line solid-phase extraction-liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A* **2001,** *939* (1-2), 1-11.
106. Ghanem, A.; Bados, P.; Kerhoas, L.; Dubroca, J.; Einhorn, J., Glyphosate and AMPA analysis in sewage sludge by LC-ESI-MS/MS after FMOC derivatization on strong anion-exchange resin as solid support. *Anal. Chem.* **2007,** *79* (10), 3794-801.
107. Office, U. G. P., U.S. EPA Method 6800 in Test Methods for Evaluating Solid Waster, Physical/Chemical methods SW846, Update IVA. Washington, DC, **2008**.